Antioxidants in Critical Illness

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Oxidative stress has been implicated in the manifestations of critical illnesses, including ischemia and reperfusion injury and systemic inflammatory states. This review describes the evidence for increased oxidative stress in critically ill patients and explores the data regarding antioxidant therapy for these conditions. Antioxidant therapies reviewed include *N*-acetylcysteine, selenium, vitamins E and C, superoxide dismutase, catalase, lazaroids, and allopurinol. We focus on the results of these interventions in animal models and human trials, when available.

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Increasing evidence supports the role of systemic oxidative stress in the development and manifestation of critical illness. *Oxidative stress* is defined as a state in which the level of toxic reactive oxygen intermediates (ROI) overcomes the endogenous antioxidant defenses of the host. Oxidative stress can result, therefore, from either an excess in oxidant production, or depletion of antioxidant defenses. Reactive oxygen intermediates are produced as a result of normal physiologic processes, including leakage of electrons from cellular electron transfer chains, and as byproducts of membrane lipid metabolism (**Figure 1**). During illness, ROI are produced by phagocytic cells as a mechanism to kill invading microorganisms. When inflammation becomes systemic, however, as in sepsis or the systemic inflammatory response syndrome, loss of control of ROI production may lead to nondiscriminant bystander injury in the host. Reactive oxygen intermediates cause direct cellular injury by oxidative injury to cellular proteins and nucleic acids, and by inducing lipid peroxidation, which leads to the destruction of the cell membrane.

In addition to causing direct cytotoxicity, ROI also play a role as second messengers in the intracellular signaling pathways of inflammatory cells. In particular, the activation of the critical nuclear transcription factor, nuclear factor κB (NF-κB), has been induced by hydrogen peroxide and blocked by several antioxidants, including vitamin E.[1,2] Nuclear factor κB is a central transcription factor involved in the regulation of numerous proinflammatory genes, including many cytokines (tumor necrosis factor, interleukin [IL]-1, IL-6, IL-8, IL-2), hematopoetic growth factors (granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, granulocyte colony-stimulating factor), cell adhesion molecules (CAM) (intercellular CAM-1, endothelial-leukocyte adhesion molecule 1, vascular CAM-1) and nitric oxide synthase (iNOS).[3] Nuclear factor κB has been demonstrated as an important mediator in the signal transduction for both endotoxin and inflammatory cytokine-induced activation.[3] A second major transcription factor, activator protein 1 (AP-1), also seems to be regulated by changes in the redox state of the cell and can be activated by both oxidants and antioxidants depending on the cell type and on intracellular conditions.[4-6] In addition, several inflammatory genes have promotor sites for AP-1, although its role in inflammatory signaling remains less well documented than NF-κB.[4] Thus, altering the redox state of the cell may contribute to the ongoing inflammatory cytokine production and progression of systemic inflammation, leading to organ injury. This

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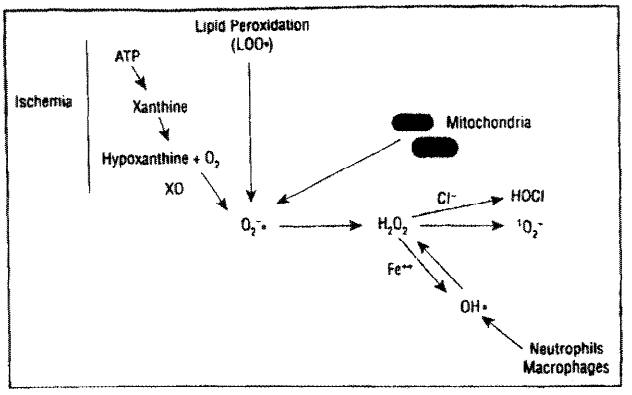

Figure 1. Sources of reactive oxygen intermediate production during critical illness. Reactive oxygen intermediates include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), singlet oxygen ($^1O_2$-), and hypochlorous acid (HOCl), all of which are produced in response to both ischemia followed by reperfusion, peroxidation of cellular membranes (LOO), and from activated phagocytic cells. Production of hypoxanthine and the activity of xanthine oxidase are enhanced by ischemia. ATP indicates adenosine triphosphate; XO, xanthine oxidase. The small square bullet denotes a free radical.

Antioxidant Defenses

Enzymatic	Nonenzymatic
Superoxide dismutase ($O_2^- \rightarrow H_2O_2$)	Vitamin E
Catalase ($H_2O_2 \rightarrow H_2O + O_2$)	Vitamin C
Glutathione peroxidase	Vitamin A/β-carotene
($GSH + H_2O_2 \rightarrow GSSG + H_2O$)	Glutathione
	Bilirubin
	Urate

may be manifest by the development of the acute respiratory distress syndrome (ARDS) or multiple organ failure syndrome.

In addition to states of systemic inflammation, oxidative stress has been implicated in the manifestations of another common cause of critical illness: ischemia and reperfusion injury. Ischemia of tissue beds followed by reperfusion with oxygenated blood, during resuscitation, leads to significant production of ROI. This is primed by the increased activity of xanthine oxidase and increased production of hypoxanthine due to loss of adenosine triphosphate during ischemia. When oxygen is reintroduced, there is both increased substrate and increased enzyme activity for the following reaction:

$$\text{Xanthine or Hypoxanthine} + H_2O + 2O_2 \rightarrow \text{uric acid} + 2O_2^-\text{•} + 2H^+$$

Ischemia and reperfusion injury occurs, on a systemic basis, during hypovolemic shock and resuscitation. It also occurs focally in several clinical scenarios, including limb ischemia with revascularization or fasciotomy, myocardial infarction with thrombolysis, and following organ transplantation.

To combat the threat of oxidative stress, there exists a number of endogenous antioxidant defenses. These include vitamins E and C, provitamin A (β-carotene), glutathione, superoxide dismutase and catalase, bilirubin, urate, and other plasma proteins. These antioxidants can be divided into enzymatic and nonenzymatic groups (**Table**). The enzymatic antioxidants include superoxide dismutase, which catalyzes the conversion of O_2^- to H_2O_2 and H_2O; catalase, which then converts H_2O_2 to H_2O and O_2; and glutathione peroxidase, which reduces H_2O_2 to H_2O by oxidizing glutathione (GSH). Re-reduction of the oxidized form of glutathione (glutathione disulfide) is then catalyzed by glutathione reductase. These enzymes also require trace metal cofactors for maximal efficiency, including selenium for glutathione peroxidase; copper, zinc, or manganese for superoxide dismutase; and iron for catalase.

The nonenzymatic antioxidants include the lipid-soluble vitamins (vitamin E, and vitamin A or β-carotene) and the water-soluble vitamins (vitamin C and glutathione). Vitamin E has been described as the major chain-breaking antioxidant in humans.[7] Vitamin E is a generic term encompassing a collection of tocopherols and tocotrienols obtained from plant oils. The most biologically active form is α-tocopherol. Because of its lipid solubility, vitamin E is located in cell membranes where it interrupts lipid peroxidation and plays a role in modulating intracellular signaling pathways that rely on ROI.[8,9(p371),10-12] Vitamin E can also directly quench ROI, including O_2^-, HO, and $^1O_2$. Vitamin A is a term encompassing a collection of retinols obtained in the diet primarily from dairy products, eggs, liver, and fortified cereals. β-Carotene is found in a variety of fruits and vegetables, and it provides approximately 25% of the vitamin A in Western diets. Dietary β-carotene is converted to retinol at the level of the intestinal mucosa, and it functions as a chain-breaking antioxidant.

Vitamin C (ascorbic acid), obtained primarily from citrus fruits, functions as a water-soluble antioxidant capable of broadly scavenging ROI, including the major neutrophil oxidants: HO, H_2O_2, and hypochlorous acid. Under certain circumstances, vitamin C has been shown to have pro-oxidant properties as well. For example, when combined with iron, it has been shown to accelerate lipid peroxidation, which leads to cellular membrane damage.[13] Finally, GSH, which is synthesized intracellularly from cysteine, glycine, and glutamate, is capable of either directly scavenging ROI, or enzymatically doing so via glutathione peroxidase (**Figure 2**). In addition, GSH is crucial to the maintenance of enzymes and other cellular components in a reduced state. The majority of GSH is synthesized in the liver, and approximately 40% is secreted in the bile.

The enzymatic and nonenzymatic antioxidant systems are intimately linked to one another, as illustrated in Figure 2. Both vitamin C and GSH have been implicated in the recycling of α-tocopherol radicals.[9(p269)] In addition, the trace elements selenium, manganese, copper, and zinc play important roles as nutritional antioxidant cofactors. Selenium is a cofactor for the enzyme glutathione peroxidase; and manganese, copper, and zinc are cofactors for superoxide dismutase. Zinc also acts to stabilize the cellular metallothionein pool, which has direct free radical quenching ability.[14] The complex interactions of these different antioxidant systems may imply that successful therapeutic strategies will depend on the use of a combination of various antioxidants rather than a single agent.

EVIDENCE OF OXIDATIVE STRESS IN CRITICAL ILLNESS

Numerous investigators have evaluated the systemic oxidant state of critically ill and injured patients. Surrogate by-products of membrane lipid peroxidation are elevated in the serum of several critically ill patient populations.[15-19] In addition, there is evidence of increased oxidant activity in the lungs of patients with the acute respiratory distress syndrome (ARDS) as manifest by increased myeloperoxidase activity and products of lipid peroxidation detected in the bronchoalveolar lavage fluid.[17] Measurement of antioxidant defenses has consistently demonstrated depressed plasma levels of vitamins E and C in patients with sepsis and ARDS.[15-17,20-23] Low plasma vitamin C levels have also been shown to be predictive of the development of multiple organ failure syndrome in populations at risk.[24] Similarly, glutathione levels are depressed in the plasma of patients with hepatic failure, in polytrauma patients, and in the bronchoalveolar lavage fluid of those with ARDS.[19,25-27]

A recently developed assay measuring total serum antioxidant status has also been applied to several populations of critically ill patients.[28,32] This assay is based on the inhibition by serum antioxidants of the absorbance of the radical cation 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). These studies have demonstrated mixed results; however, on the whole they support the presence of increased systemic oxidative stress and the depletion of antioxidant defenses during critical illness. As a result, several investigators have sought to evaluate the usefulness of antioxidant therapy for these patients.

ANTIOXIDANT THERAPY

N-Acetylcysteine

The most widely used antioxidant in experimental and clinical models is *N*-Acetylcysteine (NAC). Nacetylcysteine is converted, in vivo, to L-cysteine, which is used to replete intracellular stores of glutathione. The thiol group on the NAC molecule affords it direct antioxidant activity as well.[33] *N*-Acetylcysteine is an attractive agent for clinical trials, as it has been safely used in humans for several years for the treatment of acetaminophen overdose, and as a mucolytic agent in patients with obstructive pulmonary disease.[34] *N*-Acetylcysteine can be administered orally, intravenously, or as an inhalation agent. The oral administration of NAC increases GSH levels in the liver, plasma, and bronchoalveolar lavage fluid, suggesting a widespread systemic effect.[35]

Use of NAC in animal models of ischemia and reperfusion injury and ARDS has demonstrated encouraging results.[36-45] In models of acute lung injury, based on the intratracheal administration of lipopolysaccharide or IL-1, there was attenuation of pulmonary injury and a significant reduction in lung permeability and lipid peroxide production, even when NAC was administered up to 2 hours after endotoxin or IL-1 challenge.[37,42] A more recent study has demonstrated that liposomal encapsulation of NAC, administered intratracheally, leads to a prolonged protective effect in a rat model of acute lung injury.[46]

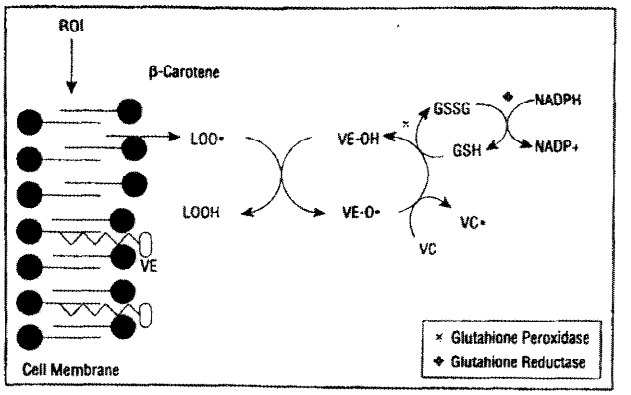

Figure 2. Interactions among antioxidants. Reactive oxygen intermediates (ROI) induce membrane lipid peroxidation, resulting in a chain reaction that can be interrupted by the direct scavenging of lipid peroxyl radicals by vitamin E (VE) and β-carotene. Both vitamin C (VC) and glutathione (GSH) can then recycle vitamin E. The reducing ability of GSH is catalyzed by the enzyme glutathione peroxidase. Glutathione is then recycled by NADPH, which is facilitated by glutathione reductase. LOO indicates active species of the lipid peroxyradical; LOOH, reduced lipid radical; VE-O, active radical form of VE; VE-OH, reduced form VE. The small square bullet denotes a free radical. (Reproduced with permission from Bulger EM & Helton WS, Nutrient antioxidants in gastrointestinal diseases. *Gastroenterol Clin North Am.* 1998; 27:403-419.)

Based on the encouraging results in animal studies, several human trials of NAC for the treatment of ARDS have been completed.[26,47-49] Recent studies of patients with ARDS have confirmed the ability of parenteral NAC administration to increase GSH levels in the bronchoalveolar lavage fluid and within pulmonary granulocytes.[50,51] Clinical trials to demonstrate benefit in patients with ARDS, however, have had equivocal results. Jepsen et al,[47] in a prospective, randomized, double-blinded trial of NAC vs a placebo in patients with established ARDS, were unable to show any difference in the PaO_2-FiO_2 ratio or survival between the groups. Similarly, Domenighetti et al[49] were unable to demonstrate any change in outcome parameters for patients with established ARDS.[49] However, Suter et al,[26] in a similar group of patients, demonstrated improved oxygenation and a decreased need for ventilatory support in the NAC-treated group. Bernard et al,[48] in a prospective, randomized, double-blinded trial of NAC, or procysteine vs placebo, were able to show an increase in red blood cell GSH levels, suggesting that the drugs were active and that the number of days of acute lung injury were significantly reduced. There was no difference in mortality in any of these studies, but all had relatively small sample sizes. Further trials are needed to determine whether patients at an earlier stage in the disease process, or preferably, those at risk for the development of ARDS, will benefit from NAC treatment.

Trials of NAC for other critically ill patient populations have also had mixed results. No overall outcome benefit was seen in a mixed population of patients in an intensive care unit.[52] Two studies of NAC administration to patients undergoing a liver transplantation have demonstrated contradictory results, with one showing no benefit, and the other showing improved liver function and better graft survival in the NAC-treated group.[53,54]

A study of the hemodynamic effects of NAC administration in patients with sepsis, revealed that 45% of patients given NAC demonstrated an increase in oxygen consumption, which was associated with an increase in gastric mucosal pH.[55] These NAC responders had a better survival rate than nonresponders. Similarly, a more recent study of NAC administration to patients with sepsis demonstrated attenuation of oxidative stress and improvement in clinical scores for these patients.[56] Lastly, NAC administration has demonstrated significant benefit in the treatment of fulminant hepatic failure secondary to acetaminophen toxicity, and it is widely used for this indication.[57,58]

Additional clinical trials with larger numbers of patients are needed to better define which population of critically ill patients may benefit from NAC therapy. In addition, it will be important to define the appropriate timing for intervention in each disease process. As suggested by the results of the ARDS trial, patients with established disease may not benefit, as the oxidant damage has been done. It may be more appropriate to target patients early in the inflammatory process or at the time of reperfusion following ischemic insults.

Selenium

Another strategy to indirectly alter the oxidant-antioxidant balance is the repletion of the trace element selenium. Selenium is a critical cofactor for the function of the enzyme glutathione peroxidase, which is involved in the oxidation of glutathione. One study has evaluated selenium supplementation in patients with systemic inflammatory response syndrome, in which all patients had low serum selenium levels at the onset of the study.[59] These authors demonstrate a lower frequency of renal failure, a more rapid resolution of organ dysfunction, and a trend toward a decreased mortality rate for patients receiving selenium supplementation. Further study is needed to fully elucidate the mechanism of benefit and clinical usefulness of this approach in different patient populations.

Vitamin E

Serum and tissue α-tocopherol levels fall steadily and dramatically in the first 24 hours following endotoxin infusion or cecal ligation and puncture.[60,61] Several investigators have demonstrated improved survival following α-tocopherol treatment in these animal models of sepsis.[62-64] In addition, α-tocopherol treatment in animals with sepsis has been shown to decrease hepatic lipid peroxidation, attenuate disseminated intravascular coagulation , and reduce plasma lactate levels.[63,65,66] Additional models of excessive inflammation in which α-tocopherol has been shown to have beneficial effects include a murine hepatic ischemia-reperfusion model, a rat renal ischemia-reperfusion model and in pulmonary inflammation following zymosan-induced peritonitis in rats.[67-69] In the liver ischemia-reperfusion study, the α-tocopherol–treated group demonstrated decreased lipid peroxidation, enhanced adenosine triphosphate generation, increased survival, and attenuation of hepatic damage.[68] In a model of renal warm ischemia, α-tocopherol pretreatment had protective effects on the kidney, as evidenced by enhanced adenosine triphosphate levels during reperfusion and lower serum creatinine levels. Increased survival was also noted in ischemic rats following treatment with α-tocopherol.[69] In the case of zymosan-induced peritonitis, administration of α-tocopherol immediately following intraperitoneal zymosan injection lead to a decrease in production of pulmonary lipid peroxidation by-products, and attenuation of pulmonary tissue damage when compared with controls.[67] This attenuation of pulmonary injury may be due to the marked inhibition of the alveolar macrophage proinflammatory response, which we have demonstrated following enteral α-tocopherol supplementation.[70]

A recent study has examined the effect of oral vitamin E supplementation on human monocyte function in healthy volunteers, who were given 1200 IU of α-tocopherol for 8 weeks.[71] Their monocytes were then harvested and found to have significantly suppressed responses to endotoxin, including decreased ROI production during the respiratory burst, decreased IL-1β production, and inhibition of monocyte-endothelial adhesion.

Despite encouraging results in animal studies and the several reports of decreased levels of vitamin E in critically ill patients, there has been only 1 clinical trial. This is likely owing to the lack of an intravenous preparation. As a result, studies are limited to the oral route, which may lead to impaired drug absorption in this patient population. One study has involved enteral vitamin E supplementation in patients with ARDS.[72] In this study, serum α-tocopherol levels, following 1-g/d supplementation, were not increased in the ARDS patients to the same degree as controls. However, it is unclear whether this was due to excessive consumption of vitamin E in these patients, or malabsorption due to severity of illness. Clearly, more well-controlled, randomized, prospective studies are needed. In addition, supplementation with higher doses of vitamin E, comparable to the efficacious animal studies, may be necessary to document a protective effect.

Vitamin C

Despite demonstration of depressed vitamin C levels in critically ill patients, supplementation with vitamin C alone has not been studied.[21,23,24] This may be because of the appropriate concern that under conditions of severe oxidant stress, vitamin C can function as a pro-oxidant by promoting iron-catalyzed reactions as an electron donor.[73] Infusion of vitamin C in patients with sepsis results in rapid consumption, due to either the promotion of redox cycling of iron or as a result of radical scavenging. There seems to be a differential handling of infused vitamin C in patients with sepsis vs healthy subjects, and further studies are needed to elucidate the relative antioxidant and prooxidant mechanisms potentially involved.[73]

Superoxide Dismutase and Catalase

Results of superoxide dismutase administration in animal models of sepsis have been variable. In general, su-

peroxide dismutase is effective when administered before the onset of sepsis,[74,76] but when administered after sepsis, it has been established that it may be harmful.[76,77] Superoxide dismutase scavenges superoxide but produces hydrogen peroxide, which requires clearance by catalase. If hydrogen peroxide is not effectively cleared, levels of the highly reactive hydroxyl radical may increase. Therefore, in this situation, superoxide dismutase may act predominantly as a pro-oxidant. Thus, it seems logical that use of superoxide dismutase therapy must include the addition of catalase administration. A potential limiting factor for both agents is their distribution. Both are large molecules, and are restricted largely to the extracellular, nonmembrane-bound space. As such, their effectiveness may be limited. Use of the 2 agents in combination has been investigated in one study of dogs with endotoxemia, and demonstrated no benefit from the combined administration whether given before or after endotoxin challenge.[78]

Combination Therapy

Based on the recognition that lipid-soluble and water-soluble antioxidants may act in a synergistic fashion, such as during the recycling of vitamin E by vitamin C, it has been suggested that a more appropriate clinical approach involves the replacement of a "cocktail" of antioxidants rather than a single agent.[79] Two clinical trials have investigated this approach. Galley et al[80] administered a combination of NAC, vitamin C, and α-tocopherol to patients in septic shock. They demonstrated a transient beneficial hemodynamic response, but did not assess the effect on outcome. A second study evaluated a supplemented enteric formulation with increased levels of vitamins E and C and β-carotene in patients with ARDS.[81] Patients on this diet required less ventilatory support, had a shorter stay in the intensive care unit, and had a decrease in the development of organ failure when compared with control patients. However, this modified diet also had alterations in the lipid content, with selective increase in the proportion of ω-3 fatty acids. Thus, it is unclear whether the benefits seen in this study are due to an increase in antioxidant activity, or to the effects of altered lipid metabolism on inflammatory cells.

Lazaroids

Lazaroids are 21-aminosteroids, which are nonglucocorticoid analogs of methylprednisolone with multiple actions, including the scavenging of ROI, the attenuation of inflammation, and the stabilization of biological membranes. Lazaroids seem promising in animal models of endotoxemia, inhalational injury, and acute lung injury.[82-86] It is likely that human trials are forthcoming. Currently, it remains unclear whether their primary effect is due to the scavenging of ROI, or modulation of the inflammatory response via inhibition of cytokine production.

Allopurinol

During ischemia and reperfusion injury, up-regulation of xanthine oxidase contributes to increased ROI production (Figure 1). Allopurinol is an inhibitor of xanthine oxidase, which has been studied as a potential therapy to down-regulate this process. Allopurinol is effective at attenuating the damage from ischemia and reperfusion injury in a number of animal models[87-90]; however, the results in sepsis models have been variable.[91,92] This suggests that the primary mechanism for free radical production in sepsis is not dependent on the xanthine oxidase pathway. Use of allopurinol in human trials has been confined to its preoperative administration to patients undergoing coronary bypass surgery, during which it has proven beneficial in attenuating the cardiac ischemia and reperfusion injury associated with this procedure.[93-95] Based on these data, studies in patients undergoing resuscitation for hemmorhagic shock are warranted.

SUMMARY

Toxic ROI play a role in the manifestations of critical illness due to both ischemia or reperfusion injury and systemic inflammation. Reactive oxygen intermediates clearly cause direct tissue injury, which can lead to organ failure. In addition, recent studies demonstrate their immunomodulatory role as second messengers within inflammatory cells. Supplemental antioxidant therapy seems promising in the regulation of the uncontrolled production of ROI in these situations. Prior to instituting this therapy, however, we must define the appropriate time points for intervention in each disease process. It seems that treatment becomes increasingly difficult as the inflammatory process and the damage induced becomes irreversible with time. In addition, we need to explore combinational therapy, as it is likely that repletion of both lipid-soluble and water-soluble antioxidants will be required. Lastly, the relatively inexpensive nature of these agents makes funding from industrial partners highly unlikely. A significant challenge lies in finding agencies willing to support encouraging therapeutics such as "simple antioxidants."

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Oxidative stress status: possible guideline for clinical management of critically ill patients

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Background. **Critical care medicine has developed in the last few years into a separate scientific discipline and studies related to the outcome after intensive care usually suggest a long hospital stay that becomes cost prohibitive. The majority of problems (death) amongst critically ill patients requiring critical care involve sepsis, inflammation, tissue damage-oxidative stress, oxygen tension PO_2, lipid peroxidation. The present investigation involves monitoring of serum levels of MDA, SOD as a possible guideline for severity of clinical situations in critically ill patients.**

Methods. **Fifty critically ill heterogeneous patients requiring intensive care in the ICU of IKDRC were selected as subjects with ages varying from 17 to 75 years. Serum levels of MDA (ng/ml), SOD (U/ml) were determined right from admission to discharge due to improvement / DAMA / death. MDA and SOD were estimated according to the methods of Buege and Aust *et al.* (1978), Nandi and Chatterji (1988).**

Results. **Critically ill patients irrespective of the disease process indicated significantly very high serum levels of MDA and low levels of SOD at the time of admission (13.28±4.26 ng/ml, 3.80±2.60 U/ml, respectively) according to the severity of the prevalent clinical situation. The pattern of serum levels of MDA and SOD according to subsequent clinical performance did indicate a decreasing trend of MDA (oxidant) and fluctuating trend of SOD (antioxidant enzyme except in those who inevitably succumbed to death in spite of adequate clinical management.**

Conclusions. **The results of the present study have amply revealed the utility and relevance of monitoring oxidative stress in critically ill patients as biochemical markers, cost-effectiveness and role in decision making (withdrawal/continuation of different support modalities) as deemed fit.**

KEY WORDS: Oxidative stress - Critical care - Malondialdehyde - Superoxide dismutase.

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Intensive care originated from the polio epidemics of 1950s Copenhagen epidemiologist Prof. Lassen.[1] Intensive care has developed over the past 30 years with little rigorous scientific evidence about what is, or is not, clinically effective. Without these data, doctors delivering intensive care often have to decide which patients can benefit most. The scoring systems based on anatomical or physiological evaluation too often have to decide which patients can benefit most. The scoring systems based on anatomical or physiological evaluation to assess severity of injury and APACHE* II and III being the most widely used scoring systems have been considered as a possible mechanism for improving resource utilisation and at the same time consideration for quality of life following intensive care.[2] In an era of escalating health expenditure and

*Acute Physiology And Cronic Health Evaluation.

TABLE I.—*Serum levels of MDA/SOD in ICU patients (mean ±SD).*

	Patients	MDA (ng/ml)		SOD (U/ml)	
		First day	Last day	First day	Last day
A	Total no. of patients (n = 50)	13.28±4.26 12.08-14.48	10.83±6.37 9.03-12.63	3.80±2.60 3.07-4.53	3.28±2.40 2.95-3.61
B	Succumbed to death (n = 16)	16.00±3.58 14.21-17.79	16.96±4.64 14.64-19.28	2.57±1.75 1.69-3.44	2.94±2.66 1.61-4.27
C	Died within 24 hrs (n = 7)	18.41±2.40 16.60-20.22	— —	3.11±1.52 1.96-4.26	— —
D	Died after prolonged stay (n = 9)	14.73±3.99 12.07-17.39	16.44±5.98 12.46-20.42	2.68±2.13 1.26-4.10	3.35±3.39 1.09-5.61
E	Recovered and discharged (n = 24)	11.70±4.01 10.06-13.34	5.65±2.91 4.46-6.84	4.12±2.85 2.96-5.28	2.95±2.21 2.05-3.85
F	DAMA (n = 10)	12.20±2.87 10.40-14.00	12.93±3.19 14.93-10.93	4.50±2.68 2.81-6.19	4.11±2.31 2.65-4.84

* confidence limit at 95%.

TABLE II.—*Statistical evolution (t values).*

	Patients	MDA		SOD	
		First day	Last day	First day	Last day
A	Total no. of patients (n = 50) df = 49	22.13 P<0.0001 HS	12.03 P<0.0001 HS	10.55 P<0.0001 HS	9.9 P<0.0001 HS
B	Succumbed to death (n = 16) df = 15	17.97 P<0.0001 HS	14.62 P<0.0001 HS	5.9 P<0.0001 HS	4.45 P<0.0001 HS
C	Died within 24 hrs (n = 7)	—	—	—	—
D	Died after prolonged stay (n = 9) df = 8	11.07 P<0.0001 HS	8.26 P<0.0001 HS	3.77 P<0.0001 HS	2.35 P<0.0001 HS
E	Recovered and discharged (n = 24) df = 23	14.20 P<0.0001 HS	9.5 P<0.0001 HS	7.10 P<0.0001 HS	6.5 P<0.0001 HS
F	DAMA (n = 10) df = 9	13.55 P<0.0001	6.46 P<0.0001	5.35 P<0.0001	5.63 P<0.0001

* HS = highly significant

increasingly constrained resources, the outcome of critical illness continues to remain a focus of attention.[3] The majority of problems (deaths amongst critically ill patients requiring critical care involve sepsis, inflammation, multiorgan dysfunction syndrome (MODS),[4] tissue damage-oxidative stress, oxygen tension pO_2 reactive oxygen species, lipid peroxidation etc. Our earlier work on oxidative stress in some clinical situations [5,6] led us to undertake present investigation with a view to understanding verity of illness and possible correlation in terms of serum levels of MDA (malondieldehyde), SOD (superoxide dismutase) representative of oxidative stress and so also its cost effectiveness as additional biochemical marker.

Materials and methods

Critically ill heterogeneous patients requiring intensive care admitted to the ICU of IKDRC were selected for the present study. Their ages varied from 17-75 years.

Malondialdehyde (ng/ml) superoxide dismutase of Buege and Aust *et al.*[7] Nandi and Chatterjee respectively right from the time of admission and subse-

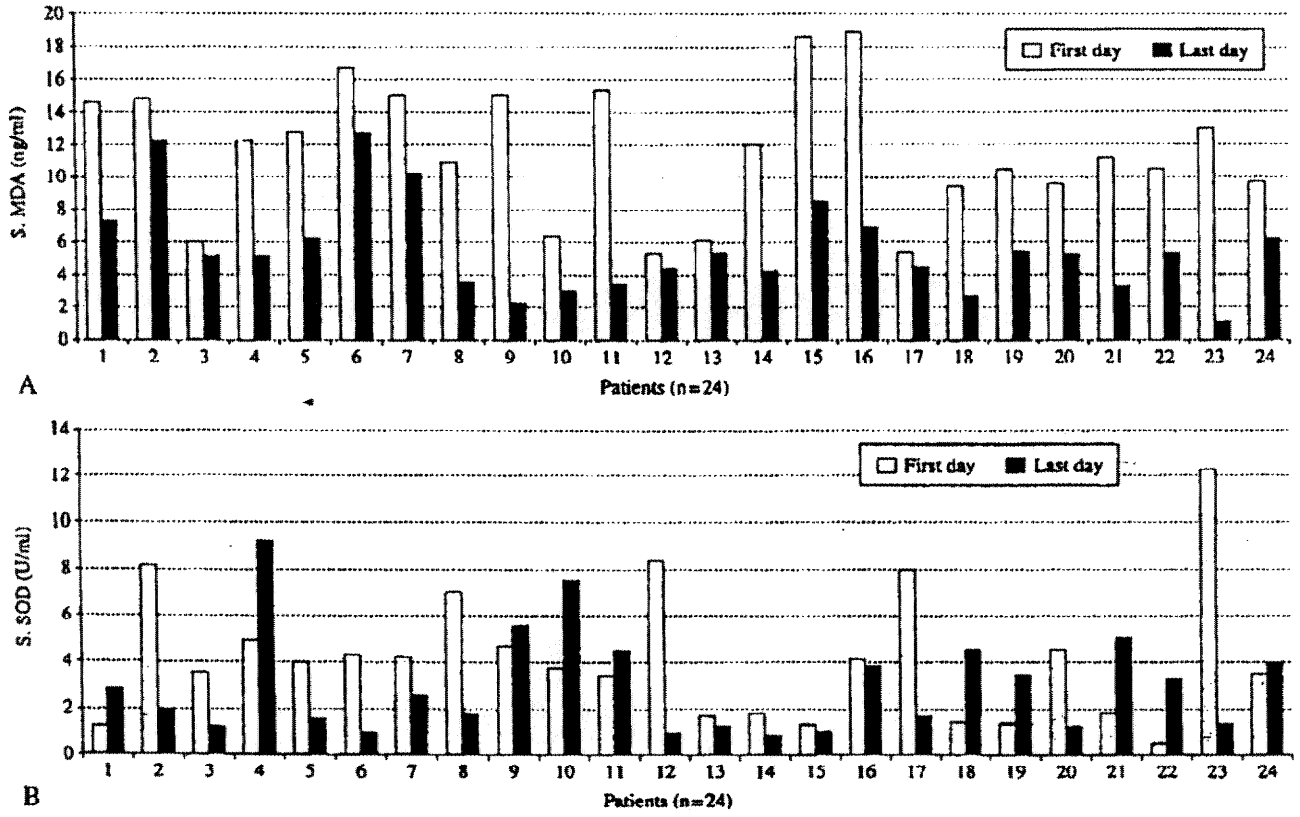

Fig. 1.—A) S.MDA levels in discharged patients (n=24); B) S.SOD of discharged patients (n=24).

quently from early morning fasting blood samples till their discharge from the ICU due to clinical improvement/DAMA*/death. Statistical evaluation was done using the paired "t" test.

Results

The results of serum DA and SOD levels are recorded in Table I and statistical evaluations in Table II. Observations *viz.* data in terms of MD and SOD of patients getting discharge following clinical recovery and those coming under category DAMA are also presented graphically in Figures 1A, B, and 2A, B, respectively. The serum levels of MDA at the time of admission and last day in ICU of different groups encountered were:

*Discharge Against Medical Advice.

A) Total patients as a whole (n=50) (13.28±4.26*, 10.83±6.37**) ng/ml.

B) Succumbed to death (n=16) (16.0±3.58*, 6.96±4.64**) ng/ml.

C) Succumbed to death within 24 hrs (n=7) (18.41±2.40*) ng/ml.

D) Succumbed to death after a week's stay (n=9) (14.73±3.99*, 16.44±5.98**) ng/ml.

E) Discharged following clinical recovery and improvement (11.70±4.01*, 5.65±2.91**) ng/ml.

F) DAMA patients (n=10) (12.20±2.87*, 12.93±3.19**) ng/ml.

The observed trend regarding serum MDA in ICU patients indicated very high levels on admission and the last day appeared to be highly significant ($p<0.0001$) in all the groups outlined above. Regarding SOD levels in these groups:

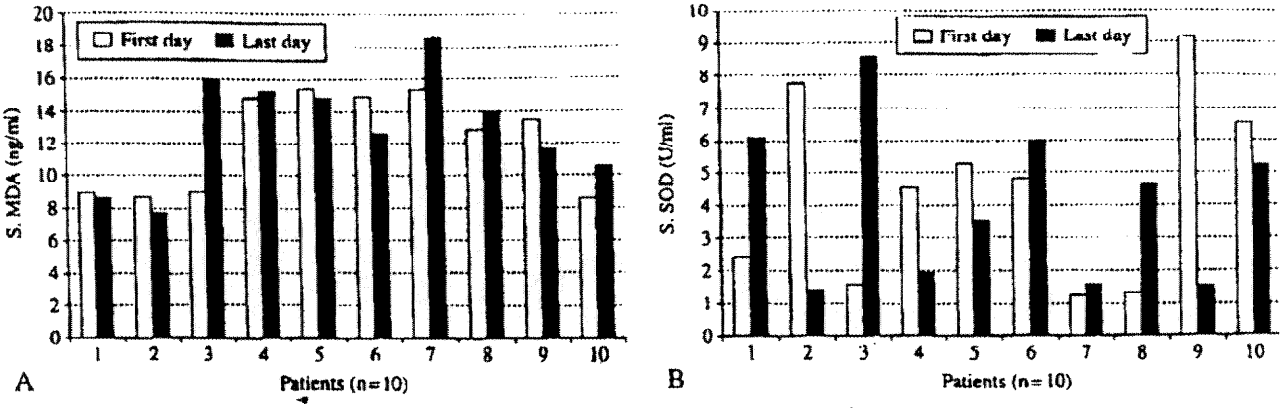

Fig. 2.— A) S.MDA levels in DAMA patients (n=10); B) S.SOD levels in DAMA patients (n=10).

A) (3.80±2.60*, 3.28±2.40**) U/ml.
B) (2.57±1.75*, 2.94±2.66**) U/ml.
C) (3.11±1.52*) U/ml.
D) (2.68±2.13*, 3.35±3.39**) U/ml.
E) (4.12±2.85*, 2.95±2.21**) U/ml.
F) (4.50±2.68*, 4.11±2.31**) U/ml.

The levels of significance in these groups (except in D group were found to be highly significant *i.e.* $p<0.0001$) whereas in D ($p<0.01$). Serum SOD alterations were significant in patients considered as a whole but otherwise they were of a fluctuating nature which can be attributed to adaptational response and immense body reserves.

Discussion and conclusions

The serious and confronting limitations of critical care are heavy economic constraints, paucity of funds and outcome following ICU care. Yet, improvement and need for extensive research work is emphasised for a better future.

Critical illness irrespective of underlying cause in our studies has confirmed the prevalence of oxidative stress as evident in terms of extremely elevated levels of serum MDA. ICU patients who succumbed to death and those who were discharged as DAMA continued to show an upward trend in MDA and fluctuating SOD levels. However, the patients getting discharged following recovery (after due intensive care) revealed a decreasing trend in serum levels of MDA/SOD as an attempt to reach physiological levels in course. Fluctuating levels of SOD (antioxidant enzyme) in ICU patients may be attributed to the prevailing number of biological stimulants *viz.* high O_2 tension, H_2O_2, endotoxin, glucocorticoid, cytokines (TNF/IL-1/IL-6).[9] These observatians do suggest a possible due consideration for antioxidant therapy, in addition to routine respiratory support, control of infection, and haemodynamic considerations.

Gutterid and Mitchell[10] observed uncontrolled production of reactive oxygen and reactive nitrogen species in their critically ill patients. Oldham and Bowen[11] reported oxidative stress status in critical care patients and emphasised antioxidant therapy as a logical answer to combat decreasing trend of antioxidant level and again cautioned that antioxidant regiment may have an adverse effect. Dasgupta *et al.*[12] observed a net decrease in the total antioxidant capacity of their ICU patients but at the same time normal hydroperoxide levels. Now as regards the outcome of our ICU patients under investigation out of 50,16 succumbed to death (7 patients within 24 hours of admission) and after a week's treatment in the ICU; and this outcome in terms of percentages is not that different from other ICUs. In conclusion, a word for relevance of oxidative stress studies and MDA, SOD as biochemical markers are relevant and useful in view of their cost effectiveness and decision making as deemed fit, taking into consideration the attitudes of both patients and their relatives.

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Review

Free radicals, cytokines and nitric oxide in cardiac failure and myocardial infarction

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Abstract

Myocardial infarction is the most common cause of congestive cardiac failure. Free radicals, cytokines, nitric oxide (NO) and antioxidants play a major role both in atherosclerosis and myocardial damage and preservation. In the early stages of atherosclerosis, neutrophils and monocytes infiltrate the intima and generate free radicals which damage the endothelial cells. As a result, production of NO and prostacyclin by the endothelial cells declines, which have cardioprotective actions. This also has relevance to the beneficial action of aspirin since, it can modulate both prostanoid and L-arginine-NO systems and NF-kB translocation. In both acute myocardial infarction and chronic congestive cardiac failure, the plasma levels of various inflammatory mediators such as interleukins and tumour necrosis factor-α (TNFα) are elevated. TNFα, produced by the inflammatory cells and the myocardium, can suppress myocardial contractility and induce the production of free radicals, which in turn can further damage the myocardium. Transforming growth factor β (TGFβ), polyunsaturated fatty acids and the glucose-insulin-potassium regimen can antagonize the harmful actions of TNFα and protect the myocardium. This explains why efforts made to reduce the levels of pro-inflammatory cytokines have beneficial action and preserve the myocardium. (Mol Cell Biochem **215**: 145–152)

Key words: cytokines, atherosclerosis, myocardial infarction, congestive heart failure, free radicals, nitric oxide, reperfusion injury, transforming growth factor β, tumor necrosis factor α, polyunsaturated fatty acids, glucose-insulin-potassium regimen

Introduction

Congestive cardiac failure is common and the most common cause being myocardial infarction. Approximately 1.5 million infarctions occur each year in the USA alone [1, 2]. Almost 25% of acute myocardial infarctions result in death, with slightly more than half of the deaths occurring before the stricken individual reaches hospital.

The recent advances that have taken place in the management of myocardial infarction such as intensive coronary care units, anticoagulation therapy, thrombolytic therapy, coronary angioplasty and bypass surgery have improved the survival of patients following hospitalization. Nevertheless, a risk of excess mortality and recurrent non-fatal myocardial infarction persists in patients who recover from an acute attack.

One of the major aims of management of the patient with acute myocardial infarction is to minimise the mass of infarcted tissue and prevent the occurrence of cardiac failure. This ensures rapid recovery and adequate myocardial function. Both thrombolytic therapy and coronary angioplasty in an acute setting aim to restore early perfusion of the ischaemic myocardium so that as much tissue as possible can be salvaged before it becomes irreversibly injured. However, reperfusion results in dysfunction of the endothelial cells of the coronary vasculature as well as injury to the myocardium termed reperfusion injury.

Nevertheless, reperfusion is desirable since appropriate thrombolytic therapy has been shown to reduce infarct size. Hence, the development of newer therapeutic strategies that aim to limit infarct size and enhance myocardial function are desirable. To develop such strategies, it is necessary to understand the molecules that play a significant role in atherosclerosis, the process which leads to coronary vascular obstruction, myocardial damage and myocardial preservation.

Free radicals, various cytokines, nitric oxide and antioxidants play an important role both in atherosclerosis and

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myocardial damage and preservation. In addition, neutrophils, macrophages and the cardiac cells themselves can produce some, if not all, of these biologically active molecules. It is certain that a better understanding of the major players in these processes may lead to the development of newer therapeutic advances in this area.

Aetiology of atherosclerosis

Atherosclerosis is the major underlying process that results in complete coronary occlusion and myocardial infarction. Major factors that can contribute to the development of atherosclerosis include: hyperlipoproteinaemia, increased platelet aggregation, damage to vascular endothelial cells and enhanced arterial smooth muscle cell proliferation. Studies both in animals and humans revealed that polymorphonuclear leucocytes, monocytes and macrophages are present in atherosclerotic lesions [1, 2].

Inflammatory cells and cytokines in atherosclerosis

High cholesterol diet induced atherosclerotic lesions contain leucocytes in clusters. Swelling of the intima of the blood vessels, which is an early marker of atherosclerosis, is known to be associated with neutrophil and monocyte infiltration of the intima. Both neutrophils and monocytes generate free radicals such as superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen, which are capable of inducing damage to the endothelial cells of the intima [2, 3]. This damage to the endothelial cells will result in reduced production of prostacyclin (PGI2) and nitric oxide (NO), which are potent vasodilators and platelet anti-aggregators. Thus, early infiltration of intima by neutrophils and monocytes may be responsible for the initiation of atherosclerosis by virtue of their capacity to generate free radicals, which are known to damage endothelial cells [2, 3]. This is supported by the observation that neutrophils of patients with hyperlipoproteinaemia release large amounts of superoxide anion compared to controls [1, 2].

LDL (low density lipoprotein) rich in cholesterol and triglycerides can enhance monocyte adhesion to endothelial cells. Under normal conditions, adherence of leucocytes to the endothelial cells is prevented by the production of PGI2 and NO by the healthy endothelial cells. Damage to the endothelial cells decreases their production and secretion of PGI2 and NO and this in turn can lead to the adhesion of leucocytes to the intima of the vessel wall.

One of the major pathways by which neutrophils and monocytes induce damage to the endothelium is through the release of free radicals. These free radicals (especially superoxide anion) can inactivate both PGI2 and NO [4], which are potent vasodilators and platelet anti-aggregators. This suggests that in the presence of inadequate amounts of NO and PGI2 platelet aggregation and vasoconstriction will occur, a situation that is conducive to the development of atherosclerosis, thrombosis and vascular occlusion, events that can lead to the occurrence of acute myocardial infarction.

At least two lines of evidence: the protective action of exercise against ischemic heart disease (IHD) and role of free radicals in atherosclerosis and myocardial damage support the hypothesis that neutrophils and monocytes and free radicals play a major role in atherosclerosis and IHD.

Mechanism(s) of cardioprotective action of exercise

Exercise is beneficial both in the prevention of atherosclerosis and IHD. Smith *et al.* [5] studied the effect of long-term exercise on atherosclerosis in persons at risk of developing IHD. In this study of subjects who exercised for a mean of 2.5 h/week the ability of mononuclear cells to produce anti-atherogenic cytokines such as interleukin-4 (IL-4), IL-10 and TGFβ rose by almost 36%. In addition, serum levels of C-reactive protein declined by 35%. These data indicate that long-term exercise decreases the atherogenic activity of mononuclear cells in persons at risk of developing IHD.

In another study, Yamashita *et al.* [6] showed that exercise significantly reduced the magnitude of myocardial infarction in rats. This cardioprotective action paralleled the change in the manganese superoxide dismutase (Mn-SOD) activity. Also, administration of the antisense oligodeoxyribonucleotide to Mn-SOD abolished the decrease in infarct size. It was also observed that simultaneous administration of the neutralizing antibodies to TNFα and IL-1β abolished the cardioprotective action of exercise and also the activation of Mn-SOD. Further, administration of TNFα showed the biphasic pattern of cardioprotection and activation of Mn-SOD as seen with exercise. These results suggest that the production of free radicals and TNFα and IL-1β induced by exercise leads to the activation of Mn-SOD which seems to play a major role in the acquisition of biphasic cardioprotection against IHD induced by exercise [6].

This is supported by the work of Wang *et al.* [7] who showed that in patients assessed by coronary angiography, the circulating extracellular-SOD (EC-SOD) is lower in men than women and in smokers of each sex and that these low levels were independently associated with history of IHD. These findings are consistent with the idea that antioxidants have protective action against IHD.

Studies by Tripathi and Hegde [8] revealed that alpha-tocopherol pretreatment significantly reduces the myocardial infarct size and percent necrosis in the left ventricular mass in comparison to the untreated animals.

It is evident from the preceding discussion that inflammatory cells, cytokines, free radicals and NO play a major role in the pathogenesis of atherosclerosis. Antioxidants such as alpha-tocopherol and endogenous antioxidant enzyme SOD may prevent atherosclerosis. The beneficial effect of exercise against atherosclerosis and IHD may lie in its ability to activate tissue levels of SOD, which can quench superoxide anion and enhance the half-life of NO. Hence, methods designed to augment tissue levels of SOD, enhance the production of NO and/or suppress the release/synthesis of superoxide anion and other free radicals may prevent atherosclerosis and IHD. In this context, administration of antioxidants such as alpha-tocopherol and recombinant human SOD both for atherosclerosis and IHD needs serious consideration.

Myocardial ischaemia and reperfusion injury

Prostanoids, nitric oxide and IHD

It is generally believed that NO and PGI2 have cardioprotective actions. Yamamoto *et al.* [9] observed that concentrations of both PGI2 and thromboxane A2 (TXA2), which has antagonistic actions to PGI2, were elevated in the infarcted portions of the heart compared to the non-infarcted regions. This increase in the levels of prostanoids was accompanied by increased activation of inducible NO synthase (iNOS). Both high (75 mg/kg/12 h) and low (5 mg/kg/day) doses of aspirin (acetylsalicylic acid) diminished the concentrations of PGI2, TXA2 and iNOS in the infarcted heart muscle whereas low doses failed to inhibit myocardial iNOS activity. This suggested a close interaction between iNOS and cyclo-oxygenase (COX) activities.

Kimura *et al.* [10] noted significant inhibition of iNOS in the infarcted portion of the myocardium at 375 and 500 mg/kg of aspirin. Since aspirin is a potent inhibitor of COX, these results suggest a close relationship between COX and iNOS activities in the heart.

This relationship between the synthesis of PGI2 and NO is supported by the work of Aitchison and Coker [11] who showed that in Langendorff-perfused rabbit hearts after 30 min of regional ischaemia and 120 min of reperfusion, the infarct size was significantly lower in hearts treated with NG-nitro-L-arginine (L-NOARG), a potent NO inhibitor compared to the control. This reduction in infarct size was abolished when the heart was co-perfused with a 10-fold excess of L-arginine, the precursor of NO [11].

On the other hand, indomethacin, a COX inhibitor, by itself did not have any effect. But, treatment with both L-NOARG and indomethacin resulted in a significant increase in infarct size compared to control. Treatment with L-NOARG alone increased PGI2 synthesis in the coronary effluent prior to the induction of ischaemia, an effect which was reversed by co-perfusion with either L-arginine or indomethacin. These results clearly indicate that the reduction in infarct size by L-NOARG could be due to increased formation of PGI2 and that there is a close relationship between the prostanoid and the L-arginine-NO system.

Anti-inflammatory agents and IHD

The earliest stages of atherosclerosis may be mediated by an auto-immune type of reaction against heat shock protein 60 (HSP60) [12]. For the interactions between HSP60 specific T cells and endothelial cells to occur, both HSP60 and certain adhesion molecules need to be expressed in endothelial cells (EC).

In a recent study, Amberger *et al.* [12] showed that aspirin is an effective suppressor of TNFα induced expression of adhesion molecule, monocyte chemoattractant protein-1 (MCP-1), on human umbilical vein endothelial cells and also inhibited the T cell proliferation in response to influenza virus antigen in a dose-dependent manner in mice. These data suggest that the anti-atherogenic effect of aspirin may be due to its ability to prevent the adhesion of sensitized T cells to stressed endothelial cells and that down-regulation of MCP-1 expression may result in decreased recruitment of monocytes to the intima.

In addition, aspirin functions as an antioxidant via its ability to scavenge hydroxyl radicals [13], and by inhibiting iNOS expression and TNFα release by cultured smooth muscle cells [14]. The anti-inflammatory effects of aspirin can also be due to its capacity to suppress TNFα gene expression in macrophages [15] and inhibition of NF-kB/c-Rel nuclear translocation in human monocytes [16, 17]. These actions may explain the beneficial effects of aspirin in the prevention of atherosclerosis and IHD.

Even cyclosporin-A seems able to reduce serum levels of TNFα and blunt the expression of cardiac intercellular adhesion molecule-1 (ICAM-1) and thus protect against myocardial ischaemia-reperfusion injury in rats [18]. On the other hand, Amberger *et al.* [12] reported that cyclosporin-A may enhance atherosclerosis in mice and that it also did not suppress TNFα induced MCP-1 expression on human umbilical vein endothelial cells. These contrasting results may in part be due to the differences in the species used in these studies.

Nitric oxide synthase, IHD and myocardial reperfusion injury

Procoagulant inflammatory responses have a role in the increased tendency for re-occlusion seen in coronary vessels following percutaneous transluminal coronary angioplasty

(PTCA) in acute myocardial infarction (AMI). This is supported by several lines of observation. For example, an increased leucocyte count is an important risk factor for subsequent adverse cardiac events in AMI. Ott *et al.* [19] studied leucocyte procoagulant activity with a 1-stage clotting assay, Mac-1 expression of monocytes and concentrations of TNFα, IL-1β, IL-6 and IL-8 in 20 patients with AMI. Systemic IL-6, C-reactive protein levels, monocyte Mac-1 expression and monocyte procoagulant activity rose significantly within 48 h after PTCA in patients with AMI compared to the control group consisting of 20 patients who underwent elective PTCA.

Reactive oxygen metabolites also participate in myocardial damage caused by ischaemia-reperfusion injury since plasma levels of lipid peroxides were raised whereas the levels of vitamins C and E were significantly lower 90 min after thrombolytic therapy in humans [20]. Surprisingly there was no correlation between the ejection fraction and lipid peroxide values at the 90th min following thrombolytic therapy which is considered to be the time of successful thrombolysis [20]. This suggests that in all probability the rise in free radical generation following thrombolytic therapy does not interfere with the cardiac function.

NO also seems to have an important role in ischaemia-reperfusion injury and IHD. Jones *et al.* [21] investigated the role of endothelial cell nitric oxide synthase (ecNOS) in myocardial ischaemia-reperfusion injury in wild-type and ecNOS-deficient mice and showed that the latter had significantly larger infarcts and more neutrophil accumulation compared to the wild type. These findings demonstrate a cardioprotective role for ecNOS derived NO in the ischaemia-reperfusion injury in the mouse heart.

On the other hand, studies by Wang *et al.* [22] suggested that NO derived from the iNOS contributes to some of the myocardial injury following IHD. However, Xi *et al.* [23] based on their studies with iNOS knockout mice concluded that disruption of iNOS gene does not exacerbate ischaemia-reperfusion injury in the heart. These apparently contradictory results can be attributed to the differences in the species used or to differences in the role of eNOS and iNOS on myocardial preservation or both. For example: Jones *et al.* and Xi *et al.* used mice whereas Wang *et al.* used Sprague-Dawley rats in their studies and to the type of NOS that was inhibited. For example Jones *et al.* studied the role of endothelial cell NOS, Wang *et al.* and Xi *et al.* looked at the role of inducible NOS. Thus, it appears that the issue of whether NO is beneficial or not is largely model dependent, dependent upon the quantity of NO present in the system, the type of NOS that is being studied and may also partly depend on how much of the free radical, peroxynitrite, is formed.

Endothelial dysfunction of the peripheral vasculature occurs in congestive cardiac failure following IHD. A study of the dilator responses, the expression of protein and mRNA of the eNOS, iNOS, soluble guanylate cyclase, superoxide anion and peroxynitrate production in aortic rings from Wistar rats after myocardial infarction demonstrated that endothelial dysfunction in IHD occurs despite an enhanced eNOS and soluble guanylate cyclase expression and is due to an increase in vascular superoxide anion production [24]. The enhanced superoxide anion production contributes to the inactivation of NO as a result of which endothelial dysfunction is seen in heart failure associated with IHD.

The importance of eNOS in the development of atherosclerosis and IHD can be gauged from the fact that its polymorphism is associated with increased susceptibility to these disorders [25]. The endothelial NOS is encoded by the NOS gene on chromosome 7. Single strand conformation polymorphism analysis of NOS 3 identified a G → T polymorphism in exon 7 of the gene which encodes a Glu → Asp amino acid substitution at residue 298 of eNOS. A study [25] on the relationship between this Glu → Asp variant and atherosclerotic coronary heart disease showed that there was an excess of homozygotes for the Asp(298) variant among patients with angiographic coronary artery disease and among patients with recent myocardial infarction compared with their respective controls [25].

Cytokines in IHD and congestive cardiac failure

Inflammatory mediators such as TNFα and ILs seem to be involved in the pathogenesis of atherosclerosis and IHD and congestive cardiac failure (CCF). Blann and McCollum [26] demonstrated increased plasma levels of TNF in survivors of myocardial infarction, which suggests inappropriate leucocyte activation. In a similar fashion, even during the phase of reperfusion, induced by thrombolytic therapy or angioplasty, neutrophils and monocytes adherent to the myocardium and intima of the blood vessels and the ischaemic and reperfused myocardium release TNF, IL-6 and interleukin-1 (IL-1), which may initiate and perpetuate reperfusion injury and enhance myocardial damage [27, 28].

Further, both TNF and IL-1 can stimulate neutrophils, monocytes, T cells and endothelial cells to generate free radicals which in turn produce more damage to the myocardium [29]. Thus, neutrophils and monocytes and the chemicals generated by them in the form of TNFα, ILs and free radicals seem to play a very important role in the pathogenesis of atherosclerosis, thrombosis and myocardial injury [1–3]. Even coronary smooth muscle cells are a potential source of TNFα during myocardial ischaemia [30].

TNF and CHF

TNFα is a known mediator in the pathogenesis of congestive cardiac failure. TNFα is secreted by adipose tissue and

plays a critical role in insulin resistance and in the pathogenesis of non-insulin dependent diabetes mellitus [31–34]. It s a mediator of inflammation and septic shock, enhances the production of reactive oxygen species including NO [29, 33]. TNF is also needed for tissue remodelling following acute and chronic infections. Thus, TNF has both beneficial and harmful actions.

Patients with advanced congestive heart failure (CHF) develop cachexia similar to those having chronic inflammatory or neoplastic diseases. This 'cardiac cachexia' is now recognised to be due to increase in the circulating levels of TNF [35]. There appears to be a direct relation between the circulating levels of TNF and clinical features of CHF. This is further supported by the observation that following cardiac transplantation TNF levels decrease [36]. High concentrations of circulating TNF are also found in other cardiac conditions such as acute viral myocarditis, dilated cardiomyopathy, cardiac allograft rejection, myocardial infarction and following cardiopulmonary bypass surgery [35, 36].

TNFα can directly decrease myocardial contractility in a dose dependent fashion [36]. TNFα, whose release occurs early in the course of acute myocardial infarction, induced myocardial injury and dysfunction, can be ameliorated to a significant extent by the administration of specific monoclonal antibody against TNFα [37]. TNF enhances programmed cell death (apoptosis) of cardiac myocytes in decompensated human heart. This may be so despite the enhanced expression of Bcl-2, a protooncogene, which protects cells from apoptosis [38].

In CHF, impairment in functional capacity is related not only to a reduced performance of the heart, but also to a defect in the peripheral circulation. This can be in the form of reduced peripheral vasodilatation due to endothelial dysfunction and a decrease in strength and endurance of skeletal muscle due to the atrophy of muscle fibres. Both these abnormalities can be produced by TNF.

For example, TNF can cause endothelial dysfunction by enhancing free radical generation, which, in turn, quenches NO. TNF by inducing damage to endothelial cells can trigger procoagulant activity and fibrin deposition [31]. Further, TNF causes apoptosis of endothelial cells. TNF-induced synthesis of NO in a variety of cells is both beneficial and harmful to the body. For example, NO can counteract the vasoconstriction induced by the activation of sympathetic and renin-angiotensin-aldosterone system and increased superoxide anion production that occurs and thus may be of benefit in CHF. On the other hand, NO induced by TNF may produce more vasodilatation than is necessary and cause hypotension that may be detrimental to the patient in the long run. Thus, TNF has both beneficial and harmful actions in CHF [39].

There is still no consensus as to the exact cause for this increased production of TNF in CHF. It has been suggested [28, 31] that the failing heart directly produces TNF or that blood flow reduction causes local ischaemia and hypoxia which in turn stimulates the macrophages to produce TNF [19]. The other interesting possibility is that in CHF there is increased mesenteric venous pressure which causes intestinal oedema and increased bowel permeability with a consequent rise increase in endotoxin absorption from the gut.

This increase in the circulating levels of endotoxin activates macrophages and other cells to produce TNF. This is supported by the observation that in patients with CHF CD14 levels (indicative of endotoxin-cell interaction) are raised corresponding to the elevated levels of TNF and cachexia [39]. Feng *et al.* [40] noted that in an experimental model of CHF there is an elevation in the levels of an endogenous inhibitor of NO synthesis. It is not known whether such an event occurs in patients with CHF also.

Transforming growth factor β and myocardial damage:

One of the endogenous factor that has desirable actions in preserving myocardial function and preventing cardiac damage is TGFβ, which is produced by various cells including B and T cells, macrophages, tumour cells and myocardial cells.

In animal studies, TGFβ begins to disappear from cardiac myocytes in the infarct zone within 1 h of coronary artery ligation. By the end of 6 h, there was almost no TGFβ in these myocytes [3, 41]. On the other hand, increased levels of TGFβ were found in viable myocytes of the subendocardial, subepicardial and lateral border zones. TGFβ is activated by plasmin and TGFβ in turn decreases the synthesis of tissue plasminogen activator (tPA) and increases the synthesis of tPA inhibitor [42]. Though these actions are anti-fibrinolytic, this suggests that a feedback regulation exists between fibrinolytic and anti-fibrinolytic processes.

TGFβ enhances collagen synthesis, angiogenesis and compensatory myocardial hypertrophy. Thus, it mediates scar formation following myocardial infarction and moderate the damaging consequences of reperfusion after myocardial ischaemia. TGFβ deactivates peritoneal macrophage hydrogen peroxide and superoxide anion production. TGFβ can also inhibit TNF production [3, 41]. This deactivation of reactive oxygen intermediate metabolism and inhibition of TNF production by TGFβ suggests an important anti-inflammatory role for TGFβ.

Thus, TNF and TGFβ have opposite actions on inflammation and free radical generation. Acute myocardial infarction is associated with an increase in circulating TNF, the local production of superoxide anion and other free radicals and the loss of coronary vasodilatation to agents such as NO [3, 37, 41, 43]. Animal studies revealed that TNF administration alone can mimic these changes. When given before or immediately after experimental coronary occlusion, TGFβ diminished the amount of superoxide anion in the coronary

circulation, maintained and/or restored endothelial-dependent coronary relaxation and prevented cardiac damage [41]. This protective action of TGFβ can be attributed to its anti-inflammatory and anti-TNF actions. Further, TGFβ blocked the rise in circulating TNF levels that is normally expected to occur during myocardial ischaemia and reperfusion [3, 41, 44, 45]. Since the cardiac TGFβ level falls during myocardial infarction, and as TGFβ has protective action against experimental coronary artery ligation, it is suggested that a functional deficit of TGFβ at the site of injury may underlie ischaemic injury.

It is evident from the evidence presented above that free radicals, NO, TNF and TGFβ play a significant role in the pathobiology of IHD and CHF. Since TGFβ has both anti-inflammatory and anti-TNF actions, it is ideally suited as a myocardial protective agent. Further, TGFβ can also help in the remodeling of the ischaemic and infarcted myocardium in view of its ability to enhance collagen synthesis, angiogenesis and compensatory myocardial hypertrophy.

Conclusions

It is evident from the above discussion that free radicals, NO and cytokines such as TNF, IL-1, IL-2, IL-6 and TGFβ play a vital role in atherosclerosis, CHF and myocardial infarction and in ischaemia-reperfusion injury. Based on such evidence efforts may be made to develop newer therapeutic strategies. For example, recombinant human TGFβ and its analogues can be tried in the treatment of myocardial infarction in conjunction with thrombolytic therapy. In a similar fashion, possible use of monoclonal antibodies to TNF and soluble forms of the TNF receptors which can effectively neutralize circulating TNF may also be considered in the treatment of CHF and myocardial infarction [36, 37, 41]. IL-10 is known to suppress the synthesis of pro-inflammatory cytokines including TNFα. In a recent study, Yamaoka *et al.* [46] demonstrated that in patients with CHF the circulating levels of IL-10 and the expression of IL-10 receptor on mononuclear leukocytes were higher than in control subjects. They also observed that the ratio of IL-10 to TNFα tended to be higher in control subjects than in patients with CHF. On the other hand, with lipopolysaccharide treatment the release of IL-10 was more enhanced from mononuclear leukocytes of patients with CHF than from control subjects. These results suggest that IL-10/IL-10 receptor system was activated in patients with CHF. Based on these studies, it tempting to speculate that IL-10 may also be tried in the treatment of CHF along with TGFβ.

At the other end of the spectrum, possible use of drugs that can enhance NO synthesis and inhibit free radical generation may be another mode of approach in CHF and other cardiac conditions. Polyunsaturated fatty acids seem to possess such a beneficial action. Gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid (GLA, AA, EPA and DHA respectively) can inhibit the production of IL-1, IL-2 and TNFα and enhance the synthesis and release of TGFβ under some well defined circumstances both *in vitro* and *in vivo* [47–50]. These fatty acids can also inhibit phagocyte-endothelium interactions and thus bring about their anti-inflammatory and anti-atherogenic actions [51].

In addition, a recent study showed that dietary supplementation of n-3 polyunsaturated fatty acids (EPA and DHA) significantly lowered the risk of death, non-fatal myocardial infarction and stroke in patients who had myocardial infarction [52]. On the other hand, vitamin E was ineffective. Freeman *et al.* [53] showed that fish oil, a rich source of EPA and DHA, not only decreased IL-1 production but also improved cachexia in dogs with heart failure.

Recently, The American College of Cardiology (ACC) and the American Heart Association (AHA) have recommended [54, 55] the option of giving intravenous glucose, insulin and potassium (GIK) regimen to patients with AMI, especially for those who are poor candidates for thrombolytic therapy and in whom the risk of bleeding is high. This recommendation is based on the results of several studies which found that the GIK regimen was beneficial in AMI [56–58]. It has been suggested that GIK regimen helps save the integrity and func-

Table 1. A summary of the actions of various preventive or therapeutic modalities and cytokines on atherosclerosis and myocardium

Hypercolesterolemia: Activates neutrophils and monocytes which infiltrate intima. Excess free radicals produced by these cells damage endothelium leading to reduced production of NO and PGI_2 and thus initiates atherosclerosis.
Exercise: Ability of mononuclear cells to produce anti-atherogenic cytokines IL-4, IL-10 and TGFβ increased and tissue levels of SOD enhanced.
PGI2, NO: Prevent atherosclerosis and protect myocardium.
ASA: PGI_2, NO synthesis by endothelium preserved which prevent atherosclerosis and protect myocardium.
TNFα: Increases free radical generation leading to decrease in half-life of NO. Damages intima and directly suppresses myocardium and induces apoptosis of myocardial cells.
TGFβ: Physiological antagonist of TNFα, suppresses free radical generation and thus, increases half-life of NO. Protects myocardium and prevents atherosclerosis.
N-3 fatty acids: Form precursor to PGI3, a vasodilator and platelet anti-aggregator, enhance NO production, suppress TNFα and IL-1 production and thus, prevent atherosclerosis and protect myocardium.
GIK regimen: Protects myocardium probably by inhibiting TNFα synthesis, free radical generation and by enhancing TGFβ and NO synthesis.

tion of cells once glucose and potassium are transported in by insulin. The exact mechanism(s) of its beneficial action is, however, not known. I suggested that GIK regimen acts through the suppression of TNFα production [59], since providing adequate amounts of glucose and insulin can antagonize the harmful actions of TNFα and reverse the nutritional and histopathological toxicity of sublethal doses of TNF in rats [60]. In view of this, routine use of the GIK regimen in patients with AMI and CHF needs to be explored (see Table 1 for a summary).

It is also possible that plasma levels of free radicals, NO, TNF, IL-1 and TGFβ can be measured in patients with CHF and myocardial infarction and these parameters can be used as prognostic marker(s) in predicting the outcome in them.

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Mitochondria, oxygen free radicals, disease and ageing

Sandeep Raha and Brian H. Robinson

Superoxide is generated by the mitochondrial respiratory chain. The transformation of this superoxide into hydrogen peroxide and, under certain conditions, then into hydroxyl radicals is important in diseases where respiratory chain function is abnormal or where superoxide dismutase function is altered, as in amyotrophic lateral sclerosis. In additon, these reactive oxygen species can influence the ageing process through mechanisms involving mutagenesis of mtDNA or increased rates of shortening of telomeric DNA.

LIFE ON EARTH evolved in changing milieu with respect to oxygen. Microorganisms that had arisen in a reducing environment, in adapting to increasing atmospheric levels of oxygen, faced a new problem – that of reactive oxygen species (ROS) produced as a byproduct of normal metabolism[1]. The oxygen molecule is capable of accepting an additional electron to create superoxide, a more reactive form of oxygen:

$$O_2 + 1e = O_2^- \quad (1)$$

In all biological systems from the simplest of prokaryotes to the higher mammals and plants, the donors of the extra electron are usually in the form of quinonoid or flavonoid electron carriers, although small amounts can be generated from transitional metal interaction with oxygen[1–3]. Superoxide can be produced enzymatically either by a specific cytochrome b_{558}–NADPH diaphorase complex in neutrophils or by the enzyme xanthine oxidase in many tissues following purine breakdown in anoxia[1]. Although these events happen only in response to specific circumstances, the majority of superoxide that is produced on a daily basis in most organisms comes from the mitochondrial respiratory chain and is probably produced by a nonenzymatic mechanism. Ubisemiquinone (UQ_{10}) species generated in the course of electron transport reactions in the respiratory chain donate electrons to oxygen and provide a constant source of superoxide:

$$UQ_{10}^- + O_2 = O_2^- + UQ_{10} \quad (2)$$

Superoxide itself can be toxic, especially through inactivation of proteins that contain iron-sulphur centres such as aconitase and succinate

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dehydrogenase and mitochondrial NADH-ubiquinone oxidoreductase[1,2]. Ferrous iron released during the inactivation becomes an important reactant in the Fenton reaction (see Eqn 4). Hydrogen peroxide formed by dismutation of superoxide is not in itself an oxygen radical and is relatively stable. Thus, enzymes have been evolved with the task of detoxifying these oxygen free radicals, collectively named the superoxide dismutases. There are three of them in mammalian systems: a cytosolic CuZn superoxide dismutase (SOD1), an intramitochondrial manganese superoxide dismutase (SOD2) and an extracellular CuZn superoxide dismutase (SOD3)[1]. They all catalyse the following reaction:

$$2H^+ + O_2^- + O_2^- = H_2O_2 + O_2 \quad (3)$$

This reaction also occurs chemically in aqueous solution and is probably responsible for the normal decay of superoxide radicals but at a slower rate. Physiological superoxide concentrations have been estimated to be of the order of 10^{-7} M in *E. coli* not containing SOD2, compared with 10^{-10} M or less in SOD2-replete organisms, which demonstrates the effectiveness of this enzyme in lowering superoxide in the cellular environment[1]. Although superoxide and its dismuted product hydrogen peroxide are the primary ROS derived from mitochondria, a second much more damaging species, the hydroxyl radical, can arise. It is very reactive, with an estimated physiological half-life of the order of 10^{-9} seconds[3]. It causes peroxidative damage to proteins, lipids and DNA and is formed by at least two mechanisms. The Fenton reaction forms hydroxyl radical from hydrogen peroxide in the presence of cuprous or ferrous ions:

$$H_2O_2 + Fe^{2+} = OH^- + OH\cdot + Fe^{3+} \quad (4)$$

whereas an alternative source of damage can involve the reaction of hydrogen peroxide to produce a hydroxylating function at the active site of CuZnSOD (Refs 4–6). Another very reactive species is peroxynitrite formed from superoxide and nitric oxide[1].

In this article, we will consider the mechanism of generation of superoxide in the mitochondria, the evidence relating to damage associated with free radical production in certain neurodegenerative diseases and the evidence connecting oxygen free radical production with ageing.

Physiological ROS production

The production of superoxide by the respiratory chain is estimated to be at rates somewhat less than 1% of the total rate of electron transport from NADH to oxygen[7–9]. The physiological fate of the hydrogen peroxide generated on either side of the mitochondrial membrane by MnSOD or CuZnSOD is to be processed by glutathione peroxidase (GPX) to water in a reaction that converts reduced glutathione (GSH) to oxidized glutathione (GSSG)[1,8]:

$$H_2O_2 + 2GSH = 2H_2O + GSSG \quad (5)$$

The GPX located in the mitochondria appears to be encoded by the same gene (*GPX-1*) as that in the cytosol[10]. This means that hydrogen peroxide can be effectively removed from either cytosolic or mitochondrial compartments depending on glutathione availability.

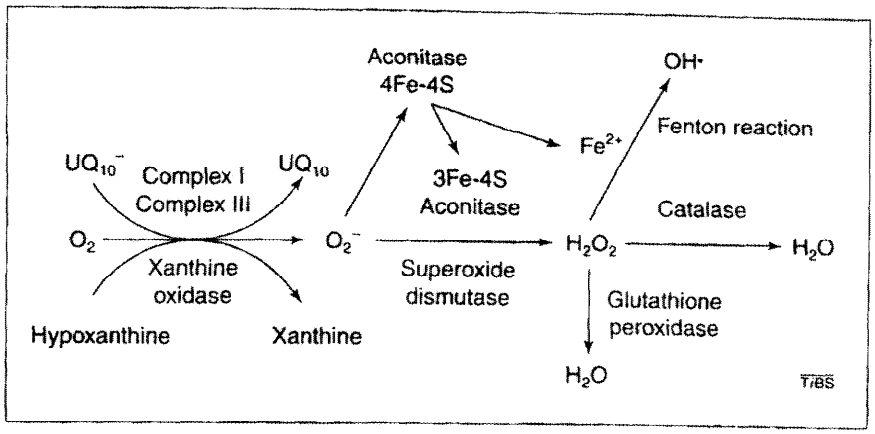

Figure 1

Reactions involved in the production and removal of oxygen free radicals in the cell. Oxygen is initially converted to superoxide (O_2^-) either by xanthine oxidase, respiratory chain complexes I and III or other cellular enzymes. The superoxide is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase. Hydrogen peroxide is converted to water by either catalase or glutathione peroxidase. In an alternative scenario, superoxide attacks enzymes (usually hydrolyases such as aconitase) with 4Fe-4S centres releasing ferrous ions. These ferrous ions participate in a Fenton reaction that converts hydrogen peroxide into hydroxyl ion and hydroxyl radical (OH·) (see text for Eqn 4).

Complex I and superoxide production

Oxidative phosphorylation in mitochondria is carried out by four electron-transporting complexes (I–IV) and one H^+-translocating ATP synthetic complex (complex V) (Fig. 1). Two of these complexes were shown to be responsible for much of the superoxide generated by mitochondria: complex I, the NADH-ubiquinone oxidoreductase, and complex III, the ubiquinol-cytochrome *c* oxidoreductase[7,8,11]. Mammalian complex I is a large macromolecular assembly of proteins comprising 34 subunits encoded by the nuclear DNA and seven subunits encoded by mitochondrial DNA (Ref. 12). A series of electron carriers that are conserved from prokaryotes through to higher mammals conduct electrons derived from the oxidation of NADH through a series of iron-sulphur centres to eventually reduce ubiquinone by a mechanism that also pumps protons from the matrix space to the outside of the mitochondrial membrane (Fig. 2). Semiquinones generated within complex I have been identified as the likely donors for transforming O_2 to O_2^-, although the exact mechanism of how and where these semiquinones are generated by the complex I components is yet to be fully elucidated[7,9]. The site or sites within complex I where semiquinones are generated are thought to be close to inhibition sites within the complex for the quinone analogue inhibitors rotenone and piericidin[7,9]. Affinity labelling of complex I by rotenone analogues has shown recently that the 20-kDa PSST subunit is the likely inhibitor-binding site, and not ND1 as was originally believed[13,14]. The proximity of this binding site to a possible 4Fe-4S cluster in PSST suggests that this subunit is the site of final transfer of electrons from the iron-sulphur proteins to quinone and a likely site for semiquinone generation. Another candidate protein for quinone or semiquinone binding in complex I is the 49-kDa subunit, because mutations present in this protein in microorganisms bestow piericidin resistance[15]. Resolution of EPR signals from semiquinones has also provided information. Two distinct species of semiquinone, designated SQ_{Nf} and SQ_{Ns}, were present in complex I (Ref. 16). SQ_{Nf}

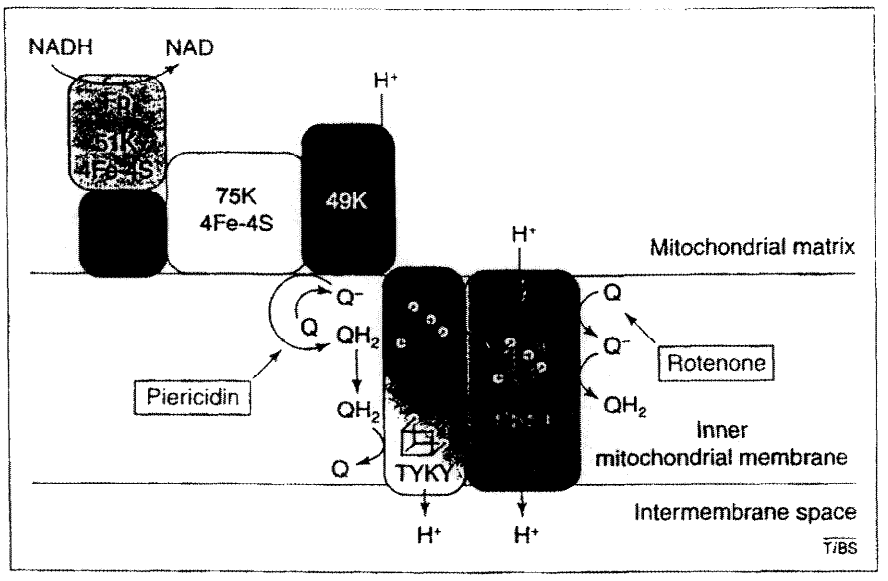

Figure 2

The generation of semiquinone intermediates in complex I. This model sets out the approximate topology of the electron-transferring subunits known to be present in mammalian complex I in relation to possible sites of ubisemiquinone generation. The initial oxidation of NADH is carried out by the flavin-containing (Fp) 51-kDa (K) subunit, which then transfers electrons to its own iron-sulphur centre. Subsequent transfers occurs through an 2Fe-2S centre in the 24-kDa subunit and at least one 4Fe-4S centre in the 75-kDa subunit. Electrons are then passed to two 4Fe-4S centres in the 23-kDa TYKY subunit and a single 4Fe-4S centre in the 20-kDa PSST subunit, both of which are associated with the membrane-spanning segment of complex I. At least two semiquinone species have been identified in association with complex I, and two suspected quinone (Q) or semiquinone (Q^-)-binding sites have been demonstrated; one on the 49-kDa subunit in the matrix arm of the enzyme and one in PSST. The model shown has two separate ubiquinone-reduction sites and one ubiquinol-oxidation site, but this could also be drawn with a single reduction site encompassing the 49-kDa and PSST subunits at the right-hand side of the complex. Separate sites for piericidin inhibition of the complex at the 49-kDa subunit and rotenone inhibition of the complex at the PSST subunit are shown, but it is possible to draw a model in which they are contiguous. Boxes depicted in the TYKY and PSST subunits show the cubic arrangement of the 4Fe-4S centres with the Fe atoms highlighted at the four corners indicated, the other corners being occupied by sulphur atoms.

interacts with the iron-sulphur cluster N2, which is thought to belong to either the 23-kDa TYKY or the 20-kDa PSST subunit[17,18]. The detailed mechanisms of how complex I operates to transport electrons linked to vectorial proton transport are not yet apparent, but superoxide production almost certainly takes place at one or both of the two semiquinone generation sites (Fig. 2). It is likely that rotenone and other complex I inhibitors increase superoxide and hydrogen peroxide production from mitochondria at complex I, possibly by blocking a site where semiquinone donates an electron to an acceptor[19,20].

Complex III and superoxide production

Complex III (ubiquinol-cytochrome *c* oxidoreductase) is responsible for taking reducing equivalents, which are generated in complexes I and II and contained in ubiquinol, and transferring them through reactions with cytochrome *b*, the Rieske iron-sulphur protein and cytochrome c_1 to the final electron acceptor cytochrome *c*. There are also two species of semiquinone generated in the mechanistic operation of complex III (Ref. 21). The Q-cycle mechanism proposed for the operation of the ubiquinol cytochrome *c* reductase operates as follows. Ubiquinol donates one electron to the Rieske iron-sulphur protein (a myxathiazol-inhibitor site) generating a semiquinone in proximity to the outer face of the inner membrane, which then reduces the first cytochrome *b* haem (b_L). The second cytochrome *b* haem (b_H) situated closer to the matrix side of the membrane accepts an electron from the first haem and reduces ubiquinone to form ubisemiquinone and, subsequently, with passage of another electron to form ubiquinol (antimycin A-inhibitor site)[21] (Fig. 3). The two inhibitors antimycin and myxathiazol, however, although they inhibit complex III electron transport equally, have dramatically different effects on superoxide production. Blocking electron passage out of cytochrome b_H prevents the semiquinone at the Q_o site from donating its electron and so, inhibition with antimycin produces a >tenfold increase in superoxide production from complex III (Fig. 3). Inhibition by myxathiazol produces little or no increase in superoxide formation because it prevents ubisemiquinone formation at the cytosolic side of the inner mitochondrial membrane[22,23]. The results of carefully examining the efficacy of various complex III inhibitors on superoxide production in isolated whole mitochondria, compared with MnSOD-depleted submitochondrial particles, also suggests that there is a sidedness to superoxide production[23]. That much of the superoxide produced by the respiratory chain under physiological conditions is generated at the matrix side of the membrane is likewise suggested by the presence of appreciable levels of MnSOD in the mitochondrial matrix and the comparatively low levels of superoxide production by whole mitochondria containing MnSOD (Ref. 23). When this enzyme is physically removed, the rates of superoxide production by mitochondria are significantly increased[23].

Damage caused by mitochondrially generated ROS

Under normal circumstances, the rate of generation of superoxide from mitochondria is rather low and does little damage, simply because it is efficiently removed by the superoxide dismutases. However, circumstances can arise for a variety of reasons (e.g. ingested chemicals that act as radical amplifiers, medically applied high concentrations of oxygen, or during periods of reperfusion of tissues with oxygen following ischaemia) where high rates of superoxide production do occur. Superoxide itself is especially damaging to the (4Fe-4S) type of iron-sulphur centre[2,3,24]. For example, damage to the cytosolic isoenzyme of aconitase evident after exposure to superoxide results in the release of ferrous ions and also sets in motion an important regulatory mechanism: the deactivated enzyme becomes a binding protein for the mRNA for ferritin, prolonging its half-life and ensuring increased ferritin synthesis to bind free iron[2,3]. The damage to other systems such as mitochondrial aconitase, complex I and succinate dehydrogenase, which also have important functional iron-sulphur centres, becomes very pronounced in MnSOD knockout mice,

where superoxide produced in the mitochondria is not removed by the normal mechanisms[24,25]. Damage to these enzymes by superoxide undoubtedly impairs the normal functioning of the citric acid cycle. This, combined with the resulting inactivation of electron transport processes, then results in disordered metabolism with lack of mitochondrial ATP generation, leading to increase flux from glucose to lactate. These mice develop lactic acidaemia, cardiomyopathy and degeneration of the basal ganglia, mimicking many of the symptoms seen in children with genetic defects of the respiratory chain[24,25]. Knockout transgenic mice lacking CuZnSOD slowly develop a neuronal axonopathy, but are not affected at birth with the severe phenotypic changes evident in mice lacking MnSOD, which suggests that either the mitochondria are more susceptible to damage from superoxide or that quantitatively intramitochondrial superoxide production is more important. On the other hand, *Drosophila* lacking CuZnSOD have a short life span[26].

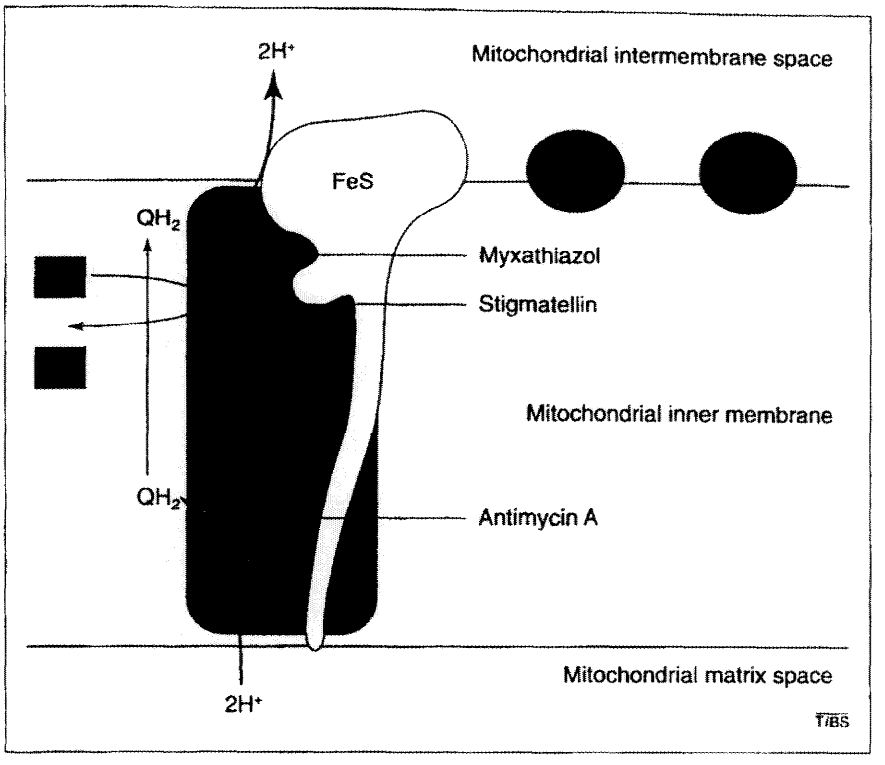

Figure 3

The production of superoxide at complex III. The figure shows the route of electron transfer from ubiquinol (QH_2) to the Rieske iron-sulphur (Q^-) protein (shown in light yellow) to produce a ubisemiquinone intermediate. This then promptly reduces the b_L haem of cytochrome *b*. The b_H haem of cytochrome *b* then reduces ubiquinone (Q) to produce another ubisemiquinone. Addition of antimycin A prevents this and results in the semiquinone generated at the Rieske protein site becoming a readily available reductant for molecular oxygen to form superoxide (O_2^-). This can be prevented by addition of either stigmatellin or myxathiazol, which prevents the formation of the ubisemiquinone at the Rieske iron-sulphur protein site.

Oxygen free radical production in human complex I deficiency

A survey of patients with inherited disorders of the mitochondrial respiratory chain showed that fibroblast cell lines from patients with complex I deficiency displayed significant elevations of MnSOD, sometimes two- to threefold, whereas patients with defects in complex III, IV or V did not[19,20,27,28]. Complex I deficiency has a wide spectrum of phenotypes varying from exercise intolerance (EI) to cataracts and developmental delay (CD), Leigh disease (LD) with degeneration of the basal ganglia, Leigh disease with cardiomyopathy (LC), cardiomyopathy with cataracts (CC) and fatal infantile lactic acidosis (FILA)[27]. A more detailed investigation showed that the patients who had the mildest symptoms (EI and CD) were less likely to induce MnSOD above the basal levels normally seen in fibroblasts, whereas patients with severe defects (CC, LD, FILA), which were usually fatal, always had elevations of MnSOD (Refs 20,27). Some patients with mild symptoms showed markedly increased superoxide production from complex I with no increase in MnSOD (Ref. 26). The correlation of increased levels of expression of MnSOD with a poor prognosis in complex I deficiency suggests that responding to the increased superoxide by inducing MnSOD could itself be detrimental in the long term because of the excess of hydrogen peroxide generated in mitochondria. High levels of the damaging hydroxyl radical were demonstrated in cell lines with increased induction of MnSOD (Ref. 28). Thus, a sequence of events resulting in severe problems could be: (i) a genetic defect in complex I; (ii) improper complex assembly or assembly of complex with abnormal properties; (iii) diversion of electrons to form increased amounts of superoxide; (iv) induction of MnSOD; (v) increased formation of hydrogen peroxide from superoxide via MnSOD; or (vi) transformation of hydrogen peroxide to hydroxyl radicals via the Fenton reaction or other mechanisms. Although these abnormalities have so far been investigated only in tissue culture, this gives a framework for future clinical investigation.

Another disease that involves misdirected oxygen free radical metabolism occurs when mutations in CuZnSOD predisposing to amyotrophic lateral sclerosis (ALS) are present[29]. Although only a small proportion of patients with ALS have such mutations, their existence has led to a number of investigations. The mutations seen in CuZnSOD in ALS patients are odd in that they do not destroy superoxide dismutase activity, even though they sometimes affect enzyme stability[5,6,29]. Instead, they seem to bestow a 'gain of function' on the enzyme. Evidence from many sources shows that these mutations result in a higher-than-normal amount of damage attributable to the action of hydroxyl radicals. How the mutant enzyme bestows this property on cells containing it is controversial[5,6]. The presence of hydroxyl radicals is associated with a rise in the titre of 8-oxodeoxyguanosine in DNA in both nuclear and mitochondrial DNA, although the latter is most severely affected, and damage might be more prominent in brain than in heart[30].

Evidence for ROS-induced mitochondrial damage associated with ageing

There is a wealth of circumstantial evidence linking the production of oxygen free radicals with the process of ageing. Graphs showing an inverse relationship in a variety of different animal species

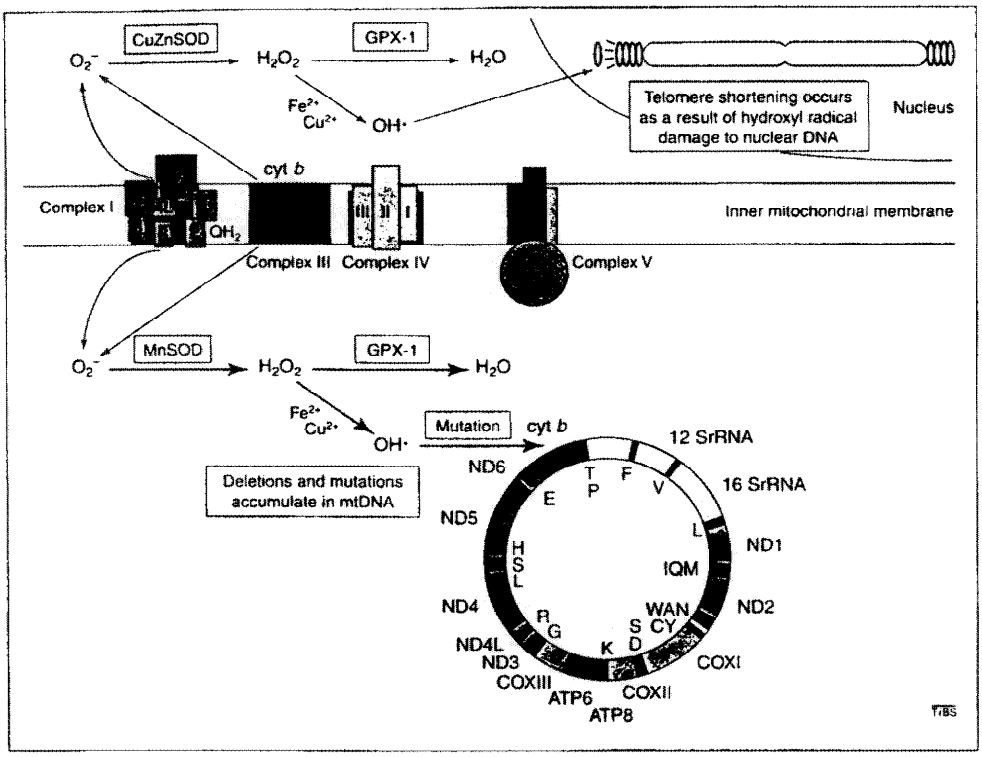

Figure 4

The generation of reactive oxygen species (ROS) by mitochondria. The mammalian mitochondrial respiratory chain is depicted as a series of complexes embedded in the inner mitochondrial membrane. Complex I (NADH-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase) and complex V (H^+-translocating ATP synthetase) are shown. Each of the four complexes is made up of subunits encoded in the nucleus and subunits encoded in mitochondrial DNA (mtDNA). For each complex the subunits encoded by mtDNA are shown in the same colour as that depicted for the encoding genes shown in the circular mtDNA to the right. MtDNA genes include those for complex I (*ND1, ND2, ND3, ND4, ND4L, ND5, ND6*), for complex III (*cyt* b), for complex IV (*COXI, COXII, COXIII*), and for complex V (*ATP6, ATP8*). Positions of tRNA genes in mtDNA, which feature prominently in mutations prevalent in human mitochondrial disease, are depicted in the circular mtDNA by the single-letter amino acid code corresponding to the specificity of the tRNA. Superoxide (O_2^-) produced at either complex I or complex III of the respiratory chain appears at both faces of the inner mitochondrial membrane. If produced at the outer face, it is processed to hydrogen peroxide (H_2O_2) by CuZnSOD, if in the matrix space, by MnSOD. Removal of the hydrogen peroxide in both compartments is achieved by glutathione peroxidase (GPX-1), which exists both in the cytosol and in the mitochondrial matrix. The formation of hydroxyl radicals (OH·) from hydrogen peroxide can proceed by the Fenton reaction in the presence of free iron or copper ions that act as catalysts. The hydroxyl radicals then can damage lipids, proteins or DNA. MtDNA has a tendency to accumulate deletions and mutations, which can then impair mitochondrial function, which can, in turn, result in higher rates of superoxide production. Alternatively, damage to telomeres in the nucleus can have an accelerating effect on ageing by increasing the shortening of telomeric DNA that occurs per unit of cell division.

between life span and (i) expression of CuZnSOD (Refs 31,32), (ii) basal metabolic rate[33], (iii) mitochondrial production[34] and (iv) mitochondrial hydrogen superoxide production[35], are compelling in that they suggest that animals with more rapid rates of basal metabolism have faster rates of ROS production and a shorter life span[30,34,35]. There is strong evidence that damage to proteins and DNA is cumulative with age, and that mutations and deletions, especially in mitochondrial DNA, are much more frequent in the aged compared with the young of most species[30]. In addition, caloric restriction in mice has the effect of lowering basal rates of metabolism resulting in a longer life span and preserved mitochondrial respiratory activity[36]. All of these data can be interpreted as indicating that life span is influenced by mtDNA deletions and mutations accumulated through exposure to oxygen free radical damage, and that this is a function of the indigenous rate of electron transport mediated by the fixed electron-transport complexes and the mobile electron-transport intermediary ubiquinone[34,35]. The faster the rate of metabolism, the faster is the rate of electron transport and the rate of generation of superoxide, leading to free radical damage to proteins, lipids and DNA by superoxide or its downstream reactive metabolites. Cells become damaged when subject to such 'oxygen stress', a word that can be used to describe the above phenomena but also can be used more freely to describe any applied increased burden of oxygen free radicals. An example of this are experiments where cultured cells are subjected to exposure to hydrogen peroxide, alkyl peroxides or free radical amplifiers such as paraquat[5,6,34]. When this stress is applied, cells will often go into a form of apoptosis. In transgenic mice bearing CuZnSOD mutations known to cause ALS in humans, the protective bcl-2 protein when overexpressed transgenically can protect against neuronal death brought about by ALS mutations in experimental animals[37,38].

One intriguing example of the link between the ROS-generating function of the respiratory chain and life span is that provided by the clk-1 protein. The clk-1 protein is responsible for one of the final steps in ubiquinone synthesis, so that defective activity results in low endogenous synthesis of ubiquinone for the mitochondrial respiratory chain. Such defective activity results in longer life span in *C. elegans*[39]. Although the crucial experiments involving superoxide production have yet to be done, the lack of ubiquinone would have the effect of slowing down oxidative metabolism, reducing the rate of ubisemiquinone formation, thereby markedly reducing the rate of superoxide production from mitochondria[40]. If life span has some dependency on oxygen free radicals, any mechanism leading to decreased free radical production ought to prolong life span. However, ubiquinone is also used widely as an anti-oxidant because of its ability to accept electrons, so, in the final analysis, a fine balance must be struck between its pro- and anti-oxygen free radical properties.

Overexpression of superoxide dismutases

Because the damage inherent from oxygen free radical production by the mitochondrial respiratory chain can be ameliorated by the judicious application of anti-oxidants and anti-oxidant enzymes, it has been postulated that more of these

enzymes would, by necessity, reduce damage and therefore might actually prolong lifespan[41]. Although this was shown to be true for overexpression of CuZnSOD in fruitflies[28,41], experiments on transgenic mice have been disappointing in this regard. The presence of increased CuZnSOD, at the same time as catalysing more efficient removal of superoxide, leads to more rapid synthesis of hydrogen peroxide, which itself can be damaging. Thus, transgenic *Drosophila* with increased expression of CuZnSOD did not show increased life span, unless accompanied by increased expression of catalase to remove the hydrogen peroxide. Such experimental procedures prolonged *Drosophila* life span by ~40% with either increased CuZnSOD expressed in all tissues or confined to the central nervous system[28,41]. In mammals, increased life span by transgenic modulation of levels of superoxide dismutase has not been observed[42,43]. Increased production of hydrogen peroxide in the cytosolic compartment or perhaps subsequent production of OH radical by the Fenton reaction has been theorized as being responsible for making the CuZnSOD-overexpressing mice more susceptible to radiation, infection and other stresses[42–44]. The overexpression of MnSOD in cultured rat glioma cells made those cells more sensitive to damage by radiation and carcinogens[45,46], whereas MnSOD overexpression in mouse heart is protective against adriamycin-induced cardiotoxicity[47]. Clearly, overexpression of superoxide dismutases is as problematic as underexpression.

Reactive oxygen species – direct mediators of ageing or agents responsible for modulation of a more fundamental ageing process

That oxygen free radicals can contribute in some way to the ageing process is suggested by a number of experimental observations, as discussed above. However, the maximum variance in mammalian systems that can be elicited from manipulation of oxygen free radical production is an extension of life span of ~40%[36,48,49]. Clearly, the role of ROS in ageing needs to be put into context with respect to other mechanisms that are known to be connected with life span, particularly the phenomenon of telomere shortening with age. Telomeres are long stretches of tandemly arranged TTAGGG repeat structures flanking the ends of each chromosome, which are necessary for successful replication[50]. Shortening of telomeres occurs by removal of DNA repeat sequences in a regulated fashion at every cell division[50]. Repeated cell divisions are possible until the extent of the telomere shortening reaches a limit[50,51]. At this point, proliferation is no longer possible, growth arrest occurs and cells go into a senescent mode. As a result of this process, there is a strong association between the number of DNA repeats present in the telomeres and the age of the individual or the number of cell divisions that have been experienced in a cell culture[51–53] (Fig. 4).

The number of repeats present in a newborn of any species is not a function of longevity of that species. That is a function of how fast the cells divide and how much telomeric shortening occurs per unit of time. It is a fact that telomere shortening seems to occur in finite increments of telomere DNA removal per unit cell division, but this could be governed in some way by the metabolic rate of the organism, or it could be influenced by oxygen free radical production from mitochondria. Human skin fibroblasts treated with DNA-alkylating agents or with hydrogen peroxide were examined for telomeric damage. Although DNA damage occurred with both reagents, the alkylating agent damage was quickly repaired, whereas that induced by hydrogen peroxide was not[51,53]. Thus, the telomere length was reduced significantly by peroxide-induced damage, suggesting that ROS actively attack telomeric DNA and cause increased shortening, as DNA loss from the telomeres was increased from 30 bp per cell doubling to as high as 90 bp per cell doubling[51,53,54]. What is most convincing is that the telomeres are radically shortened in the premature ageing syndrome of progeria[50]. Although the primary genetic cause of this disease is not known, it has been observed that cells from progeria patients have low levels of primary antioxidant enzymes such as glutathione peroxidase, again suggesting a link between oxygen free radicals and the ageing process[55].

All of this evidence points to a basic ageing mechanism, governed by telomere shortening, which can be modulated by the lifetime production rates of oxygen free radicals. The mechanism of influence seems to be mediated through fragility of the telomeric sites predisposing the DNA to single-strand breaks and other damage. When reactive oxygen species are responsible for such damage, the breaks cannot easily be repaired and telomere shortening is accelerated[53,54] (Fig. 4). Dietary restriction or mild hypoxia, either by reducing overall electron flow through the respiratory chain or by decreasing the amount of oxygen available to react with semiquinones, could cause deceleration of telomere shortening and thus extension of life span. Interference with ubiquinone synthesis in some organisms, as demonstrated by the *C. elegans* clk-1 mutants, would be expected to cause a reduction in ubisemiquinone levels, a reduction in mitochondrial superoxide production and a subsequent deceleration of shortening and increase in life span[40].

The understanding of this relationship between mitochondria, free radical production and the rate of telomere shortening is at an early stage. A new round of experimental work is needed to explain the exact nature of these relationships between mitochondrial metabolism, mitochondrial free radical production, basal metabolic rate, mtDNA mutations and deletions, telomeric damage in chromosomal DNA and the whole complex process of ageing.

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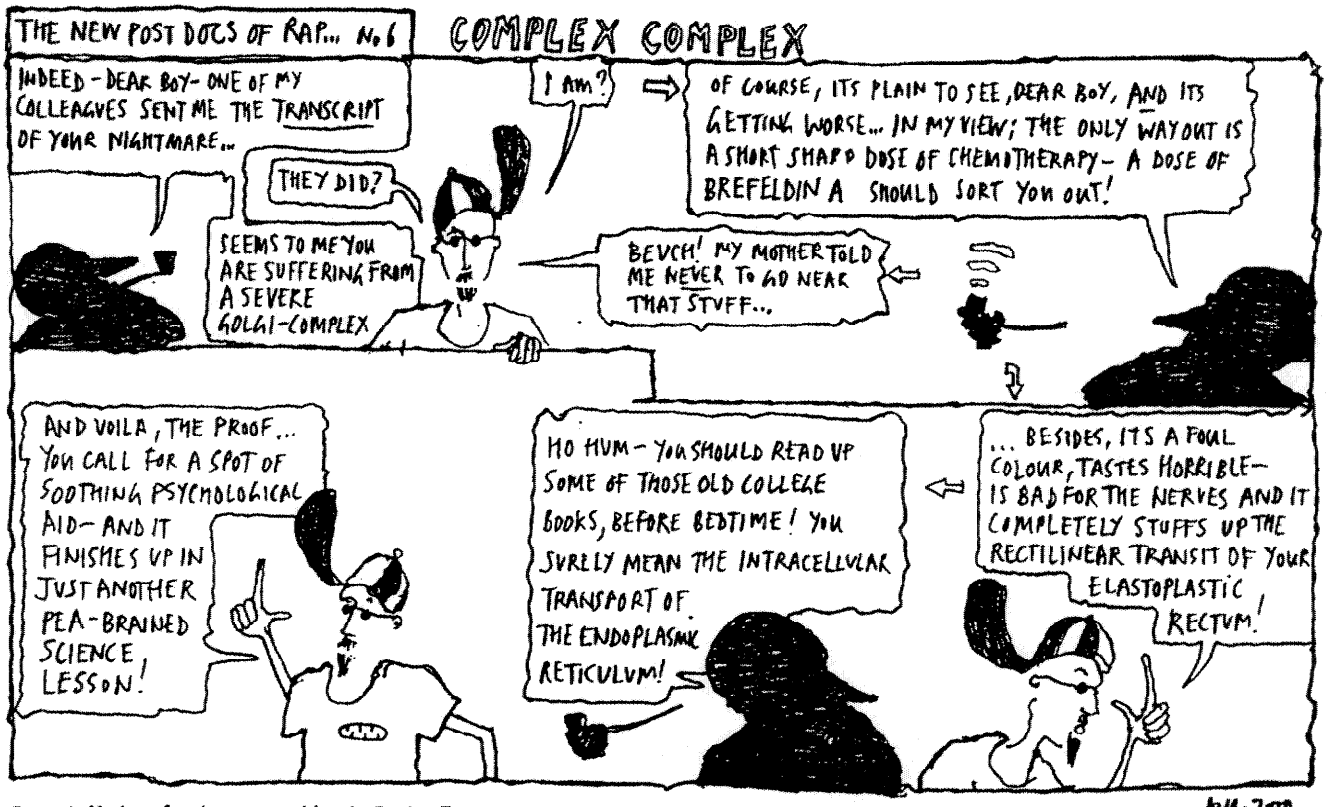

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Intensive Care Medicine

Biochemical concepts

Oxygen-free radicals and lipid peroxidation in adult respiratory distress syndrome

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Abstract. Activated species of oxygen have been implicated as mediators of some acute lung injury. In adult respiratory distress syndrome (ARDS), polymorphonuclear leukocytes accumulate in the lung and release excessive amounts of O_2-derived products into the extracellular environment. The effects of these O_2 products on lung tissue are multiple. In particular, they can initiate lipid peroxydation in cellular membranes. Excessive lipid peroxydation in membranes destroys cells such as vascular endothelium. Lipid peroxides are also detrimental to cellular functions. Lipid peroxydation could then play a role in the pathogenesis of ARDS.

Key words: Oxygen free radicals – Lipid peroxidation – Lung injury

ARDS is a catastrophic illness characterized by an interstitial and alveolar edema, by hypoxemia due to the intrapulmonary shunting of blood, by a decreased pulmonary compliance and increased permeability of the pulmonary microvasculature. Several hypotheses have been considered as potential causes of ARDS: activation of complement and coagulation factors, aggregation of platelets and leukocytes, release of endogenous vasoactive substances. It appears that oxygen radicals released from polymorphonuclear leukocytes play a central role in certain forms of ARDS [1] among these causes.

Oxygen-free radicals are unstable chemical species characterized by the presence of unpaired electrons in their outer orbit. These highly reactive substances are extremely toxic [2]. Oxygen radicals can damage cell membranes causing lipid peroxidation [3] and they can degrade hyaluronic acid in the interstitial space [4]. Their overproduction in the lungs, especially during an inflammatory reaction, is thus able to injure the alveolar-capillary membrane.

Also the lipid-free radicals are unstable chemical species. They are derived from peroxidative degradation of polyunsaturated fatty acids which are localized in biological membranes. This peroxidative decomposition of cell membrane lipids is a chemical event which could be of importance in the development of damage of the pulmonary endothelial barrier. This review aims to show the importance that lipid peroxidation could have in the pathogenesis of ARDS.

Oxygen free radicals

Under normoxic conditions, most of the oxygen that diffuses into the cells is reduced to H_2O by cytochrome oxydase localized in the mitochondria. It is the complete, tretavalent reduction of oxygen which is used to generate adenosine triphosphate [2]. However, 1% to 2% of oxygen undergoes a univalent reduction (Fig. 1) which produces first superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) (2,5); the superoxide radical is then able to react with H_2O_2 to form a hydroxyl radical (Haber-Weiss reaction) and

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} OH^- \xrightarrow{e^- + H^+} H_2O$$

$$2O_2 + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

$$2H_2O_2 \xrightarrow{Catalase} O_2 + 2H_2O$$

$$H_2O_2 + 2GSH \xrightarrow[peroxidase]{GHS} GSSH + 2H_2O$$

$$ROOH + 2GSH \xrightarrow[peroxidase]{GHS} ROH + H_2O + GSSH$$

Fig. 1. Univalent pathway of oxygen reduction and the enzymatic mechanisms for the removal of the toxic oxygen intermediates. Superoxide dismutase (SOD) and catalase catalyze the dismutation reactions for O_2 and H_2O_2, respectively. Glutathione peroxidase (GSH) eliminate hydrogen peroxide and lipid peroxides

singlet oxygen (O_2^*). O_2^- may react also with lipid hydroperoxides to form alkoxy radicals (RO') (6). O_2^- and H_2O_2 are relatively innocuous but their destructive potential consists in being able to induce hydroxyl radicals. These elements are powerful unstable oxydants and the major damaging factors in vivo. Singlet oxygen is also highly reactive and an important mediator of tissue damage. The superoxide radical is produced in the cells [2] during the auto-oxidation of numerous compounds by certain oxidative enzymes. Leukocytes also generate large amounts of superoxide anion during the respiratory burst which accompagnies phagocytosis [7] to kill bacteria and viruses.

Usually, reactive oxygen intermediates cause little problem and are normal transients. In an organism, there is a balance between free radical generation and neutralisation by endogenous defense systems which protect the integrity of cells and tissues. Superoxide radicals are scavenged by superoxide dismutase which catalyze their conversion to hydrogen peroxide [5]; hydrogen peroxide is detoxified by catalases and oxydases which convert it to H_2O_2 and O_2 (2). These intracellular defense systems thus prevent the formation of the reactants O_2^- and H_2O_2 which are required for the production of OH^-.

However, if the defense systems are deficient or if free radicals are generated at a rate exceeding the antioxidant capacity of the organism, free radical reactions will be highly toxic and dangerous to tissues. If the activated polymorphonuclear leukocytes e.g. produce superoxide anions in large amounts into the extracellular space, these free radicals will initiate highly deleterious chain reactions causing cellular and tissue damage. Moreover, as there is very little superoxide dismutase in the extracellular space, the damaging effects of O_2^- released into the extracellular space will be largely unopposed.

Oxygen free radicals and lipid peroxidation

Uncontrolled production of oxygen radicals in the intracellular and extracellular spaces can provoke a great variety of, mainly destructive, reactions [8]. In particular these highly reactive agents stimulate the process of lipid peroxidation in biological membranes [9]. This radical-induced lipid peroxidation involves an initiation step in which a polyunsaturated fatty acid (RH) interacts with a reactive oxidizing radical (X') (Fig. 2).

This interaction results in the abstraction of a hydrogen atom to form a lipid radical (R') which rapidly reacts with molecular oxygen to generate a lipid peroxy free radical (ROO'). This lipid, containing a fatty acid peroxy free radical, extracts a hydrogen atom from an adjacent lipid (RH) to form a lipid hydroperoxide (ROOH) and a second lipid radical (R'). The lipid peroxy free radical can also cyclize to form an endoperoxide radical. Finally, lipid hydroperoxides and endoperoxides undergo a decomposition leading to the formation of numerous stable products including carbonyls, malondialdehyde and volatile hydrocarbons such as ethane and pentane [10, 11]. Arachidonic acid is one of the most important polyunsaturated fatty acids of cellular membranes which may undergo a peroxidative degradation. Normally it is subjected to rigidly controlled free radical reactions to produce endoperoxides and hydroperoxides. The enzyme cyclo-oxygenase catalyzes the hydrogen abstraction, the oxygen insertion and the rapid cyclization to form prostaglandin endoperoxides [12]. The enzyme lipoxygenase catalyzes the initial step of hydrogen abstraction and the oxygen insertion to form a peroxy radical which is further protonated to produce the hydroperoxide. This hydroperoxidation of arachidonic acid produces several isomers of hydroperoxy fatty acid which may be further metabolized to leukotrienes [13].

The interaction between oxygen free radicals and cell membrane fatty acids thus results in the generation of lipid peroxide radicals, lipid hydroperoxides, lipid endoperoxides and degradation products. Lipid peroxidation is also the first step of the generation of arachidonic acid metabolites.

Lipid peroxidation is normally prevented by antioxidants which include enzymes removing H_2O_2 and O_2^-, by alpha-tocopherol and β-carotene, and glutathione peroxidase which reacts directly with lipid peroxides [10].

The cellular consequences of a lipid peroxidation [10, 14] which overhelms intracellular protective processes are believed to be importat causes of cell membrane destruction. They stem from plasma membrane alterations and from the release of hydroperoxides and malondialdehydes. In the plasma membrane, lipid peroxidation leads to an alteration of the physical properties of phospholipids [10, 11]. There is a perturbation of the structure of the membrane and a loss of interaction between proteins and fatty acids resulting in an inactivation of some enzymes like Na-K ATPase, adenylate cyclase and cytochrome oxydase.

$$X^* + RH \longrightarrow R^* + XH$$
$$R^* + O_2 \longrightarrow ROO^*$$
$$ROO^* + RH \longrightarrow ROOH + R^*$$

Fig. 2. Mechanism for lipid peroxidation. X' = free radical; RH = lipid with a methylene hydrogen; R' = lipid radical; ROO' = lipid peroxy radical; ROOH = lipid hydroperoxide

Moreover, lipid peroxidation leads to a lowering of membrane fluidity [11, 15], which could favour entrapment of cells such as platelets and leukocytes on endothelial cells.

All these physical modifications of membrane lipids alter the functional integrity of the membrane which results in an increased permeability [11].

Fatty acid hydroperoxides are toxic for the capillary endothelium in the lungs. Anderson et al. [16] have shown that intravenous injection of fatty hydroperoxides produce stripping and fragmentation of the endothelial plasma membranes. These endothelial alterations are associated with the development of interstitial and alveolar edema. Lipid peroxides also have been shown to be inhibitors of prostacyclin synthetase decreasing the ability of the endothelial cell to produce prostacyclin [17]. Consequently, lipid peroxides may play a role in platelet aggregation. Hydroperoxides are also inflammatory mediators with a chemotactic activity. Several of these chemoatractant hydroperoxides, especially the 'hydroperoxy-eicosatetraenoic acid (HPETE), are derived from arachidonic acid [18, 19]. Finally, hydroperoxides are inhibitors of membrane-bound enzymes [10]. Malondialdehyde (MDA) is an end-product of lipid peroxidation. It is reactive toward sulfhydryl and toward the amino groups of proteins resulting in an inactivation of enzymes, in an inhibition of DNA, RNA, and protein synthesis [20].

Oxygen free radicals, lipid peroxidation and ARDS

After some insults, the lung becomes the target organ of an inflammatory reaction which, uncontrolled, may be toxic for the lung [21]. All inflammatory reaction includes acute recruitement of inflammatory cells, release of multiple humoral factors, changes in vascular calibre and flow, and increased vascular permeability with edema. A common feature of classic inflammatory reactions is the accumulation of polymorphonuclear leukocytes which play a fundamental role. A leukostasis in the lung has already been demonstrated in hemorragic shock [22], after burn trauma [23], in sepsis [24], in hyperoxic lungs [25], after microembolization [26] and during extracorporeal circulation [27, 28].

In these diseases, pulmonary sequestration of stimulated leukocytes in microvasculature and their emigration across the endothelial lining leads to a release of toxic oxygen radicals, proteolytic enzymes and inflammatory mediators which enhance the local inflammatory response (Fig. 3). During the respiratory burst of these activated inflammatory cells, large amounts of oxygen radicals are produced into the extracellular space [7]. This is not unique to the polymorphonuclear leukocytes; alveolar macrophages also are inflammatory cells responsible for a release of toxic oxygen products [29, 30].

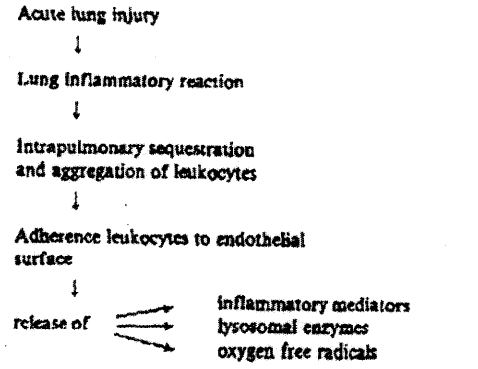

Fig. 3. Lung inflammatory reaction which may result in respiratory failure

These oxygen free radicals are cytotoxic to endothelial cells [31, 32] which are potential targets because of their close association with leukocytes during the inflammatory reaction, the outer membranes of the polymorphonuclear leukocytes are apposed to the endothelial cell membranes [33, 34]. There is an adherence of the leukocyte membrane to the endothelial surface [35]. Now, it is well known that the site of formation of superoxide radicals is not only in the mitochondrial space but also and especially in the outer surface of the membrane of leukocytes [36, 37]. An

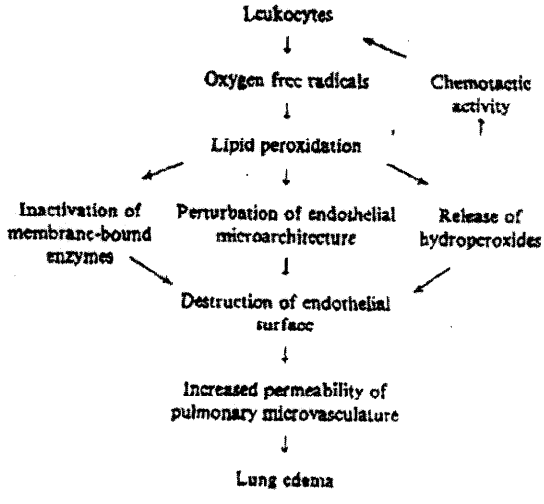

Fig. 4. Proposed role of lipid peroxidation in acute respiratory failure

NAPDH oxidase localized in the plasma membrane of leukocyte is activated, leading to the release of superoxide anions into the extracellular space [2, 38]. However, as extracellular spaces are deficient in superoxide dismutase and catalase, oxygen radicals released and accumulated in those spaces are able to cause damage to surrounding tissues. The role of these oxygen free radicals in the pathogenesis of acute edematous lung has already been demonstrated [39]. Nevertheless, the mechanisms by which oxygen radicals may contribute to acute lung injury remain speculative. One of the possibilities for oxygen radicals to damage lungs is suggested by the observation that all of these radicals may attack the polyunsaturated fatty acid side-chains of membrane lipids initiating peroxidative chain reactions. The consequence of this membrane lipid peroxidation is a disruption of cellular membranes with release of intracellular components such as lysomal enzymes and with release of hydroperoxides, endoperoxides and other prostaglandins. Lipid peroxidation initiated by free radicals thus provokes the loss of the functional integrity of the endothelial cell barrier. As free radicals degrade hyaluronic acid [4] and extracellular fibronectin [40, 41], two essential structural components, the endothelial barrier and the interstitial space are finally damaged with increased permeability and pulmonary edema [Fig. 4].

In summary, after some pulmonary insults with intense inflammatory reaction, free radicals liberated by granulocytes react with cellular membranes and initiate chain reactions. Lipid peroxidation is one of these chain reactions involving membrane lipids which has already been demonstrated in some acute lung injuries. Exposure to hyperoxia, nitrogen dioxide and ozone initiates production of lipid peroxides in lung [42–44]. Lipid peroxidation in lung may also occur with the herbicide paraquat [45]. However, the role played by lipid peroxidation in other causes of lung injury remains to be elucidated [46]. This lipid peroxidation could be identified and measured by the analysis of tissue malonaldehyde and exhaled hydrocarbons. The measurement of exhaled hydrocarbons would be a noninvasive method of monitoring in vivo lipid peroxidation in human subjects with lung injury [47]. The established lethality of ARDS on the one hand and the therapeutic possibilities for opposing lipid peroxidation on the other hand justify further studies in this field.

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LIPID PEROXIDATION AND GLUTATHIONE PEROXIDASE ACTIVITY IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE EXACERBATION: PROGNOSTIC VALUE OF MALONDIALDEHYDE

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ABSTRACT

Results of recent studies have indicated that during exacerbation of chronic obstructive pulmonary disease (COPD), antioxidant capacity is lower and the levels of lipid peroxidation products are higher than those in age-matched healthy subjects. The aim of this study was to assess the time course of changes in oxidant stress during the treatment of exacerbation of COPD. For this purpose, we measured erythrocyte glutathione peroxidase (GP_x) activity and serum levels of the lipid peroxidation product malondialdehyde (MDA) in 18 male patients with acute exacerbation of COPD. Fifteen healthy non-smokers having no history of lung disease served as control subjects. Mean erythrocyte GP_x values of patients were 45.54±9.04 u/gHb on admission and had increased to 72.77±9.68 by the tenth day of treatment, but still remained lower than those of healthy subjects (83.13±10.91) ($p=0.007$). Serum MDA values in patients were

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significantly higher (2.68±1.28 nmol/ml) than those in control subjects (1.04±0.36 nmol/ml) (p=0.000) and returned to normal values by the tenth day of treatment (1.08±0.36 nmol/ml) (p=0.766). Erythrocyte GP_x values in patients who were current smokers (39.87 ±3.82 u/gHb) were lower than those in ex-smokers (49.15±9.67 u/gHb) (p=0.021). Moreover, serum MDA values in patients who were current smokers (3.32±1.18 nmol/ml) were higher than those in ex-smokers (1.66±0.60 nmol/ml) (p=0.007). The results show that oxidative stress in patients with acute exacerbation of COPD is related to higher MDA levels that return to normal conditions during the course of treatment. In conclusion, the results suggest that MDA levels can serve as a marker of prognosis and of the success of treatment of the exacerbation of COPD.

KEYWORDS

chronic obstructive pulmonary disease, lipid peroxidation, glutathione peroxidase

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a significant health problem worldwide /1, 2/. Although many studies have been done on the protease-antiprotease theory in the pathogenesis of COPD, especially emphysema, very few have investigated the imbalance between the oxidant/antioxidant systems. Because the numbers of active neutrophils and macrophages increase in the airways of smokers and COPD patients, more superoxide anion ($O_2^{\cdot -}$) radical is released from their cells than from cells of nonsmokers or healthy subjects /3–6/.

Rahman and colleagues /7/ demonstrated that the plasma anti-oxidant capacities of the patients in acute exacerbation of COPD were lower than those in healthy subjects and stable COPD patients,

whereas the level of the lipid peroxidation product malondialdehyde (MDA) was higher. In the present study, we aimed to assess the changes in markers of oxidative damage during the treatment of patients with exacerbation of this disease.

MATERIALS AND METHODS

Subjects

This study was carried out on a control group of 15 male healthy nonsmokers (brothers or cousins of patients) and 18 male patients who were hospitalized for acute exacerbation of COPD, diagnosed according to the standards of the American Thoracic Society /1/. As a criterion of hospitalization, we used the European Respiration 'Intensive acute attack criteria in COPD' for selecting the 18 patients.

COPD treatments

The drugs used by the patients before hospitalization were such agents as inhalant steroids, inhalant B_2 agonists, mucolytics, and/or ipratrophium bromide. After hospitalization, the patients were treated with nasal oxygen therapy (2 to 4 l/min), antibiotics (except those who had no leukocytosis), mucolytics (N-acetylcysteine), inhalant B_2 agonists, parenteral or oral and later inhalant steroids, ipratrophium bromide, and oral or parenteral theophylline. Whereas 7 patients were active smokers, 11 patients had quite smoking for more than 6 weeks (from 6 weeks to 10 years).

Determination of serum MDA and GP_x

Blood samples were drawn three times (before treatment, at 48 h, and at 10 d after the beginning of treatment) from patients who were

hospitalized for an acute COPD attack. Blood samples were drawn only once from healthy control subjects. None of the patients smoked during hospitalization. Serum MDA was determined spectrophotometrically using the thiobarbituric acid (TBA) reactivity method /9/. Malondialdehyde, an end product of fatty acid peroxidation, consists of a colorful complex, giving a maximum absorbance at 532 nm after reacting with TBA. Using an extinction coefficient of the MDA-TBA complex at 532 nm, we calculated the MDA level in nmol/ml. The determination of erythrocyte GP_x was accomplished using a Randox™ kit, based on the method of Paglia and Valentine /10/. Enzyme activity was calculated as u/gHb by multiplying the dilution by the coefficient 41 and then dividing by the value of the hemoglobin control.

Statistical analysis

Statistical evaluation of the results was done with the Student's *t* and the Mann-Whitney U tests, using the SPSS computer program.

RESULTS

The average age was 59.11±3.10 for the study group and 59.33±3.02 for the control group; no statistical difference between the groups (t=0.21; p=0.84) was found. The average serum hemoglobin concentration of the patients was 15.79±0.85 g/dl. Arterial blood gas measurements for PaO_2 and $PaCO_2$ were determined as 51.51±6.52 mmHg and 43.26±3.80 mmHg, respectively. The mean FEV_1 was 39.24±5.90 (% of expected value).

The erythrocyte GP_x and serum MDA levels of the patients are shown in Table 1. The erythrocyte GP_x activities of patients with exacerbation of acute COPD were significantly lower than those of healthy control subjects (t=10.82; p=0.0000). Serum MDA levels of patients before treatment were significantly higher than those in healthy control subjects (t=4.80; p=0.000). Despite increased GP_x levels

TABLE 1

Erythrocyte GPx and serum MDA levels in patients treated for COPD and in healthy control subjects

Assay	Admission	48 hours	10 days	Controls
GPx (u/gHb)	45.54±9.04	54.20±10.53	72.77±9.68	83.13±10.91
MDA (nmol/ml)	2.68±1.28	1.27±0.39	1.08±0.36	1.04±0.36

levels during treatment, we found that the tenth day erythrocyte GP_x activity was still lower than that of healthy control subjects (t=2.89; p=0.007).

Although a significant difference between serum MDA levels in the healthy control subjects and in patients 48 h after treatment (t=1.76; p=0.088) was seen, no significant difference was found between levels in patients on the tenth-day after treatment and levels in healthy control subjects (t=0.30; p=0.76).

Whereas the erythrocyte GP_x activities of COPD in active smokers were lower than in those who had quit smoking (p=0.021), the serum MDA levels of active smokers were higher than those in subjects who had quit smoking (p=0.007) (Table 2). To determine the efficacy of treatment in active smokers and in cases who had quit smoking before beginning treatment, we compared the pre- and post-treatment levels in smokers and ex-smokers. As seen in Table 2, a statistically significant recovery in the levels of erythrocyte GP_x and serum MDA levels after treatment occurred in both groups. From this result, we concluded that the treatment of the acute exacerbation of COPD has a positive effect on the oxidant-antioxidant system.

TABLE 2

Serum MDA and GPx levels before treatment and on day 10 of treatment in active smokers and in cases who quit smoking

	GPx		p =	MDA		p =
	Admission	Day 10		Admission	Day 10	
Smokers (n=7)	39.87±3.82	62.41±8.52	0.0006	3.32±1.18	1.28±0.32	0.0017
Ex-smokers (n=11)	49.15±9.67	73.71±9.28	0.0000	1.66±0.60	0.77±0.21	0.0003

DISCUSSION

An imbalance in the oxidant-antioxidant system plays an important role in the pathogenesis of COPD /11/. The plasma antioxidant capacity of smokers and persons suffering from acute COPD exacerbation is lower than that of healthy individuals from the same age group having the same plasma anti-oxidant capacity /12/.

In patients with COPD, Postma and coworkers /13/ showed a correlation between $O_2^{\cdot}{}^-$ release by peripheral blood neutrophils and bronchial hyperreactivity. Infections may contribute to oxidative stress in COPD patients by facilitating the recruitment and activation of phagocytic cells in the lung /14/. *Haemophilus influenzae* and *Streptococcus pneumoniae* emerge during remission and exacerbation periods of COPD /15/. These pathogens can stimulate oxidative damage bound phagocytic cells. Nevertheless, $O_2^{\cdot}{}^-$ production decreases to normal levels during the period when COPD is stable, despite its high levels in the exacerbation period. Rahman and colleagues /7/ reported that $O_2^{\cdot}{}^-$ production by neutrophils increases in COPD patients during acute exacerbation and then returns to normal in the recovery period. Similarly, Muns and colleagues /16/ demonstrated

that the oxidative burst of peripheral blood phagocytes in patients with stable COPD is no different than that in healthy subjects.

In the study of Rahman and colleagues /7/, plasma antioxidant capacity, measured as Trolox equivalent antioxidant capacity (TEAC), was low at the time of admission in patients with acute exacerbation of COPD. The plasma antioxidant capacity remained low during the 48 h following admission, but increased significantly by the time of discharge. Serum MDA levels in patients with COPD at the time of presentation were higher than those in normal subjects. Twelve hours after admission, MDA decreased and remained low until discharge. During the same study, plasma antioxidant capacity, in accordance with the results of another study /12/, was lower in smokers than in persons who had quit smoking.

In the present study, erythrocyte GP_x activity was lower and the serum MDA level was higher in COPD patients at the time of presentation as compared with those in control subjects. Despite a treatment-induced increase in GP_x activity, we found that the tenth-day GP_x activity was still lower than that of the control subjects. Besides, an important decrease in the level of serum MDA occurred after the treatment and remained low, similar to control values, until discharge. The erythrocyte GP_x activity in heavy smokers suffering from COPD was lower than that in subjects who had quit smoking, but the level of serum MDA was higher, in accordance with the results of previous studies /7, 12/.

The reason for the improvement in the antioxidant capacity at the end of the acute COPD exacerbation treatment is not definitely known. Prednisolone causes and increase in antioxidant capacity, due to the stimulation of thiols like glutathione synthesis in the liver /17/. Steroids can expose an antioxidant influence, thereby reducing the oxidative and chemotactic reply besides the number of neutrophils. Evidence has been presented /18/ that a daily intake of oral steroid for a long time decreases the production of $O_2^{\cdot -}$. Another study determined that a decrease in the level of $O_2^{\cdot -}$ is produced by polymorphonuclear (PMN) leukocytes in patients suffering from emphysema who receive prednisolone treatment /19/.

Different studies have shown that β-agonists have certain antioxidant influences. For example, in patients suffering from chronic bronchitis and under formoterol and terbutaline treatment, the level of $O_2^{\cdot -}$ produced by alveolar macrophages decreases /20, 21/. Nevertheless, the data are incompatible with the influences of theophylline on oxygen radicals produced by PMN leukocytes /22–24/.

Antibiotics that are used for treating chronic bronchitis would seem to have a certain role in reducing oxidative stress in COPD by reducing infection and thereby lung inflammation /25/. Although given initially because of it mucolytic properties, N-acetylcysteine is a thio-containing compound that may act as an antioxidant by providing cysteine intracellularly for the enhanced production of glutathione /26, 27/.

In conclusion, the results presented here show that in patients with acute exacerbation of COPD, oxidative damage is related to a lipid peroxidation that returns to normal conditions during the course of treatment. Thus, MDA levels may be an important marker showing the success of treating such exacerbation. Additionally, assessing the possible role of any specific medications like steroids, β_2-agonists, theophylline, and others on the oxidant/antioxidant capacity in the treatment of COPD exacerbation requires further studies in a larger group of patients.

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Diabetologia

The importance of lipid-derived malondialdehyde in diabetes mellitus

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Abstract

Malondialdehyde (MDA) is a highly toxic by-product formed in part by lipid oxidation derived free radicals. Many studies have shown that its concentration is increased considerably in diabetes mellitus. Malondialdehyde reacts both irreversibly and reversibly with proteins and phospholipids with profound effects. In particular, the collagen of the cardiovascular system is not only stiffened by cross-links mediated by malondialdehyde but then becomes increasingly resistant to remodelling. It is important in diabetes mellitus because the initial modification of collagen by sugar adducts forms a series of glycation products which then stimulate breakdown of the lipids to malondialdehyde and hence further cross-linking by malondialdehyde of the already modified collagen. Some progress is being made into the mechanisms of formation and the nature of the intermolecular cross-links induced by malondialdehyde which result in the stiffening of the collagenous tissues. Our recent studies indicate the formation of pyridyl cross-links. Malondialdehyde has been shown to react several orders of magnitude faster with the pre-existing collagen enzymic cross-links than the amino acid side-chains. Malondialdehyde modification of basic amino-acid side-chains also results in a change in properties, for example, in the charge profile of the molecule resulting in modified cell-matrix interactions. Although aspects of the biochemistry of malondialdehyde are still not fully understood its complex chemistry is being unravelled and this should lead to ways of preventing its damaging reactions, for example, through antioxidant therapy. [Diabetologia (2000) 43: 550–557]

Keywords Malondialdehyde, cross-linking, diabetes, atherosclerosis.

Introduction

Malondialdehyde (MDA) is generated by both lipid oxidation and as a by-product of prostaglandin and thromboxane synthesis. Its plasma concentration is increased in diabetes mellitus and it is found in the atherosclerotic plaque deposits promoted by diabetes [1]. It is also found in Alzheimer's disease [2], with effects that extend far beyond the lipid field.

Oxidation of complex lipids in vivo is largely caused by oxygen-derived free radicals (OFR) such as $OH^{\cdot}$. These radicals are formed by lipoxygenases as a response to cell injury, typically from H_2O_2, or a metal-ion radical complex. The major targets of these damaging species are the long-chain polyunsaturated fatty acids of cellular phospholipids, which are particularly prone to attack because of the arrangement of double and single bonds. The resultant lipid peroxide frequently decomposes to a radical [3], which reacts with most biological molecules, including proteins and lipids. Further decomposition of these lipid peroxides produces toxic aldehydes, in particular 4-hydroxynonenal (mainly from linoleic acid) malondialdehyde

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Abbreviations: MDA, Malondialdehyde; OFR, oxygen-derived free radicals; ox-LDL, oxidised LDL; NPO, N^{δ}-(2-pyrimidyl)-L-ornithine; β-LAA, N^{ϵ}-β-lysyl-aminoacrolein; LML, 1,3-di(N^{ϵ}-lysino)propane NLMDD N-lysyl-4-methyl-2,6 dihydropyridine-3,5-dicarbaldehyde.

(mainly from arachidonic acid) [4] and acrolein, whose sources are not yet fully characterised [5]. The liver eliminates MDA from the circulation by the action of aldehyde dehydrogenase and thiokinase such that injected MDA has a half-life of approximately 2 h in rats, but some (10–30%) must bind semi-permanently to proteins as it is not eliminated within 12 h [6]. The toxicity of MDA arises from its high reactivity, particularly towards proteins and DNA.

Under normal circumstances the extent of lipid oxidation is largely controlled by antioxidant concentration in the surrounding medium which is usually sufficiently high to prevent propagation of oxidative free radical reactions by OFR in blood. In tissue, there is, however, a greater likelihood that localised deficiencies of antioxidants would allow lipid oxidation to occur. This has led to a huge interest in dietary antioxidants and their protective role in cardiovascular disease [7]. In addition to OFR, glycated collagen has also been shown to increase the oxidative breakdown of lipids compared with normal collagen [8] which is one explanation for the increased concentrations of MDA in serum and tissues in diabetic subjects.

The major carrier of lipid in blood is low density lipoprotein (LDL), a potent risk factor for coronary heart disease (CHD). When sufficient MDA modifies the protein (apolipoprotein B100) of circulating LDL, it no longer reacts with the normal LDL receptor in hepatic and peripheral cells, but only with scavenger receptors of macrophages [9]. Thus, if less than 12% of lysine side-chains are modified, the LDL is still recognisable to its receptor. If more than 15% are modified, however, only the scavenger receptor can recognise the LDL [10]. The clinical relevance of the reaction between MDA and proteins is highlighted in atherosclerosis, a disease prevalent in developed cultures, in which the arterial intima becomes infiltrated with "foam cells", lipid-laden macrophages, resulting in thickened, non-resilient arteries with reduced blood flow [11]. This is a major cause of CHD and strokes. Malondialdehyde-LDL, in addition to oxidised LDL (oxLDL) mediates several pro-inflammatory and pro-atherogenic processes, all of which ultimately lead to foam cell generation [12]. It is important to realise that the term "oxidised LDL" has habitually come to be recognised as any LDL with some degree of modification caused by lipid peroxidation and can range from the so-called minimally modified product which can be recognised by the LDL receptor to the fully modified protein which is recognisable only to the scavenger receptor on macrophages. Malondialdehyde is a major, but not the only, modifying agent and has been used frequently as a model compound for making in vitro "ox LDL".

Malondialdehyde also reacts rapidly with other proteins. It has been shown to cross-link bovine serum albumin to form dimers and also modifies RNase, crystallin, and haemoglobin [13–15]. Such adducts diminish these proteins' susceptibility to proteases and they are formed much faster when the protein is already glycated by sugars [16]. These modified proteins are targeted by antibodies and removed from the bloodstream. Malondialdehyde can also react with DNA, having been shown to be both mutagenic and carcinogenic.

Our own particular interest is the high reactivity of MDA with the long-lived proteins of the vascular system, collagen and elastin. Due to the slow turnover of these proteins in the extracellular matrix and the crystallins of the eye, this eventually becomes a problem as they become cross-linked and resistant to proteolysis. We have shown that the glycation cross-linking of the aortic collagen is related to the mechanical stiffening of the aorta of diabetic subjects [17]. Similarly, the physical properties of aortic elastin have increased stiffness and a loss of basic groups with consequent conformational rearrangements [18]. The precise mechanisms are still not clear, encouraging new biochemical studies characterising the nature of the additional MDA adducts and cross-links responsible for the changes in properties of the aorta.

In this review we summarise the effects of MDA on collagen in relation to the cardiovascular system and the finding of a potential biomarker of lipid-derived oxidative stress which could lead to the identification of possible treatments. Malondialdehyde is a potential major candidate for the deleterious effects of cross-linking and side-chain modification of the collagen of the vascular system. Its reactions with collagen are only now beginning to be unravelled.

Structure and chemistry of MDA

Malondialdehyde is a white hygroscopic crystalline compound and is typically obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane [19]. Radioactively labelled ^{14}C-MDA can be prepared from 1,3-propanediol using alcohol dehydrogenase [20]. It is more stable in plasma than might be expected because it enolises readily, losing a proton at neutral pH to form a salt (Fig. 1). Its reactivity is therefore very dependent on pH.

Determination of MDA

Malondialdehyde is used as a putative marker of lipid oxidation both in plasma and in arterial lesions. There is now substantial evidence that both ox-LDL and MDA occur in the atherosclerotic plaque.

Chemical measurement. Malondialdehyde reacts strongly with thiobarbituric acid producing fluorescent thiobarbituric acid reacting substances [21].

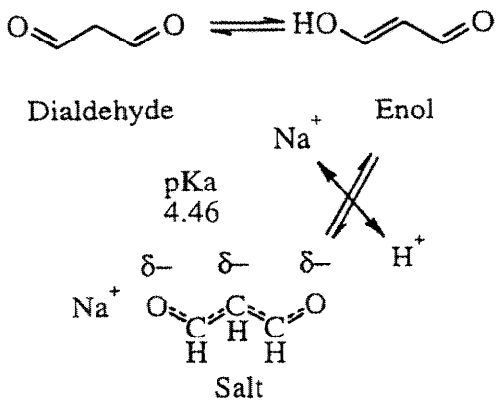

Fig. 1. Enol and salt forms of malondialdehyde

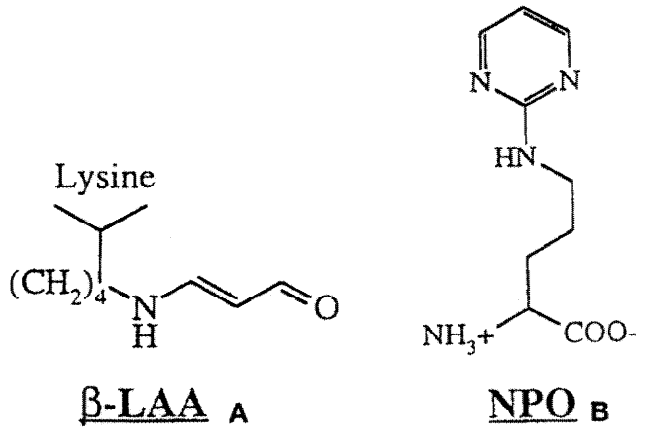

Fig. 2A, B. Reaction products of MDA with (**A**) Lysine and (**B**) arginine

This reaction, although simple and reproducible, is unfortunately rather non-specific because thiobarbituric acid reacts with many other carbonyl-containing compounds. Plasma fatty acids can also oxidise during the 95 °C heating step with thiobarbituric acid, generating artificially high results [22, 23]. The use of EDTA-containing plasma and high performance liquid chromatography coupled with post-column thiobarbituric acid derivitisation can, however, identify the specific malondialdehyde-thiobarbituric acid complex, providing a relevant assay for MDA in biological fluids [24–26].

With these precautions, normal concentrations of MDA in plasma have been shown to be in the range 0–1 μM/l. Concentrations of free MDA alone are as low as 50 nmol/l [27], but chemical work suggests that at least 80% of MDA is protein-bound. These concentrations are increased in diabetes mellitus where hyperglycaemia is known to accelerate lipid oxidation [28], partially explaining the increased risk of atherosclerosis.

Immunological measurement. The presence of both ox-LDL and MDA-LDL in the circulation is difficult to confirm directly although autoantibodies to ox-LDL have been shown in plasma and can give an indirect measure. Evidence reviewed recently suggests that the relation between circulating ox-LDL autoantibodies and risk of cardiovascular disease is inconclusive [29]. Oxidised-LDL will inevitably be a mixture of subunits with different levels of oxidation or MDA complexes or both. Assays to detect autoantibodies to ox-LDL only react with a specific antigen. For example, autoantibodies to other forms of ox-LDL will not necessarily bind MDA-LDL prepared in vitro. An additional complication is that autoantibodies to ox-LDL already complexed with ox-LDL (immune complexes) have also been detected in the circulation of diabetic patients [30]. Because the complexed autoantibodies to ox-LDL cannot react with more autoantibody this could lead to a falsely low assessment of ox-LDL antibody status when assayed by the convential ELISA method. It has also been suggested that autoantibodies which bind MDA-LDL trigger autoimmune responses [31].

Reaction of MDA with amino acids

Model reactions with amino acids. At neutral pH, MDA hydrolyses to acetaldehyde and formic acid over a few weeks, a reaction that is catalysed by the presence of amino acids such as lysine or arginine [32] but at the same time forms adducts with these amino acid side-chains. These basic amino acids are the only ones whose side-chains react with MDA to form adducts. The ε-amino group of lysine reacts with MDA to form the semi-stable N^{ε}-β-lysyl-aminoacrolein (β-LAA) (Fig. 2A), [19, 33], whereas arginine reacts roughly an order of magnitude slower to form the stable N^{δ}-(2-pyrimidyl)-L-ornithine (NPO), (Fig. 2b) [34, 35]. The side-chain of cysteine reacts with oxidised fatty acids but not directly with MDA [36]. Schiff bases formed between MDA and tryptophan or histidine are much more stable than β-LAA [33].

Mechanism of formation of potential model cross-links. Malondialdehyde is best viewed as two entirely different molecules depending on the pH. For example, at a pH below its pKa MDA reacts with lysine to give firstly β-LAA, then 1,3-di(N^{ε}-lysino)propane (LML) (Fig. 3) which can be isolated after sodium borohydride reduction of the Schiff-base adduct of two blocked lysine molecules and MDA [37]. When either the concentration of MDA is lowered to physiological levels [33] or when the pH is neutral [19], LML is, not observed unless high concentrations of sodium borohydride are present in the MDA/lysine mixture [13].

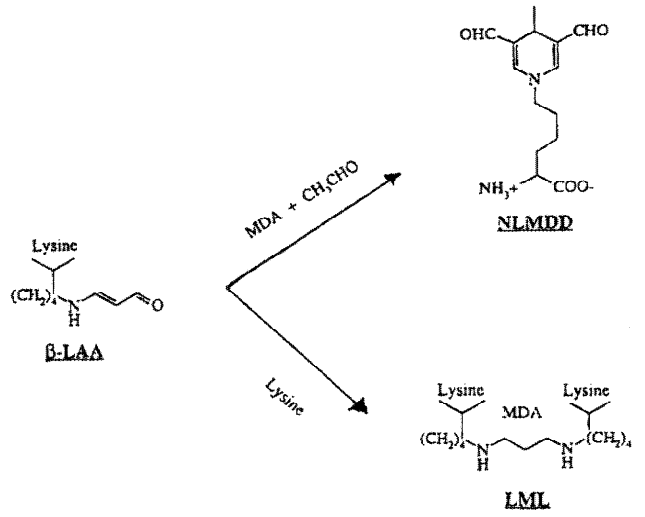

Fig. 3. Reaction of β-LAA with lysine (or arginine) to form potential intermolecular cross-links LML and NLMDD, respectively

The product of lysine with MDA, β-LAA, can, however, react further with another molecule of MDA and a molecule of acetaldehyde (derived from MDA breakdown in vitro, but present in serum) to form a stable fluorescent product, *N*-lysyl-4-methyl-2,6-dihydropyridine-3,5-dicarbaldehyde (NLMDD), (Fig. 3) [19, 31, 32]. The aldehyde side-chains on the stable dihydropyridine ring are then capable of further reactions with lysine to form a reversible cross-link [19]. Such an intermolecular cross-link would stiffen the collagen fibres of the aorta or vascular lesions.

Whether this cross-link reaction occurs under physiological conditions is still to be established. It is possible that other plasma components could react in place of the second MDA and acetaldehyde, for instance, fumarate, 4-hydroxynonenal, acrolein, sugars, and various Maillard intermediates from the reaction of sugars and proteins.

Model reactions of MDA with proteins

Protein cross-linking. Malondialdehyde has been shown to cross-link proteins by molecular weight changes and visually, for example, after a short incubation with MDA rat tail tendon becomes rigid. The collagen becomes insoluble after only 24 h and the formation of intermolecular cross-links is shown by thermal isometric tension studies and differential scanning calorimetry (Fig. 4). Polyacrylamide gel electrophoresis and column chromatography have conclusively shown, by the increase in molecular weight, that protein cross-links are formed after reaction with MDA, at least for RNase [13], α-crystallin [14] and soluble collagen (Fig. 4).

The adducts generated on incubation of protein with MDA occur essentially at random along the protein chain, preventing any direct analysis by nuclear magnetic resonance or other spectroscopic method. In an attempt to isolate these potential cross-links we have found that acid hydrolysis results in breakdown of any potential MDA cross-links. So far, development of an efficient enzyme hydrolysis procedure that reliably breaks down glycated collagen to individual amino acids and cross-links has been unsuccessful. As a result, the only method used successfully to date is the stabilisation of some of the products by sodium borohydride reduction followed by acid hydrolysis to achieve separation of the individual products. Using this technique, β-LAA has been shown to react with lysine from RNase to form an imidopropene cross-link as LML [13]. Such a protein cross-link in the Schiff base form could easily hydrolyse at neutral pH to release the lysines and MDA. There was, however, no hint of its presence in model lysine-MDA reactions analysed by nuclear magnetic resonance and it is unlikely that the β-LAA-Lysine equilibrium with LML would shift sufficiently towards LML to favour its formation in proteins. It is therefore probable that its detection is an artefact generated by the sodium borohydride reduction. Other similar investigations with bovine serum albumin and collagen using smaller quantities of sodium borohydride (but still in vast excess) have not resulted in the isolation of this link [15,19]. This could be because the conjugated Π system in LML and β-LAA is hard to reduce, requiring considerable quantities of reducing agent for successful reduction.

The most stable lysine product characterised in chemical work as a potential cross-link is the dihydro-pyridine derivative, NLMDD (Fig. 3), which is also vulnerable to acid hydrolysis even after reduction with borohydride [34]. For this reason it has not yet been isolated from proteins treated with MDA.

Effect of MDA on the pre-existing collagen cross-links. Collagen fibres are stabilised by a series of lysine-aldehyde cross-links [38] and surprisingly our recent work has shown that MDA reacts with these natural collagen cross-links orders of magnitude faster than lysine [34]. Presumably MDA cleaves the natural Schiff-base cross-links by competitive reaction with the lysines and then forms a new intermolecular cross-link. Unfortunately they again form 'putative cross-links' that are rapidly degraded by acid hydrolysis as shown by the absence of the C-14 label in the products after incubation with C-14 MDA [34], hence the nature of these cross-links is still to be explained.

In summary, despite the evidence of increasing molecular weight no cross-link derived from MDA has been isolated and characterised. A potential product has been identified (NLMDD) but not the final peptide bound product to confirm its role as a

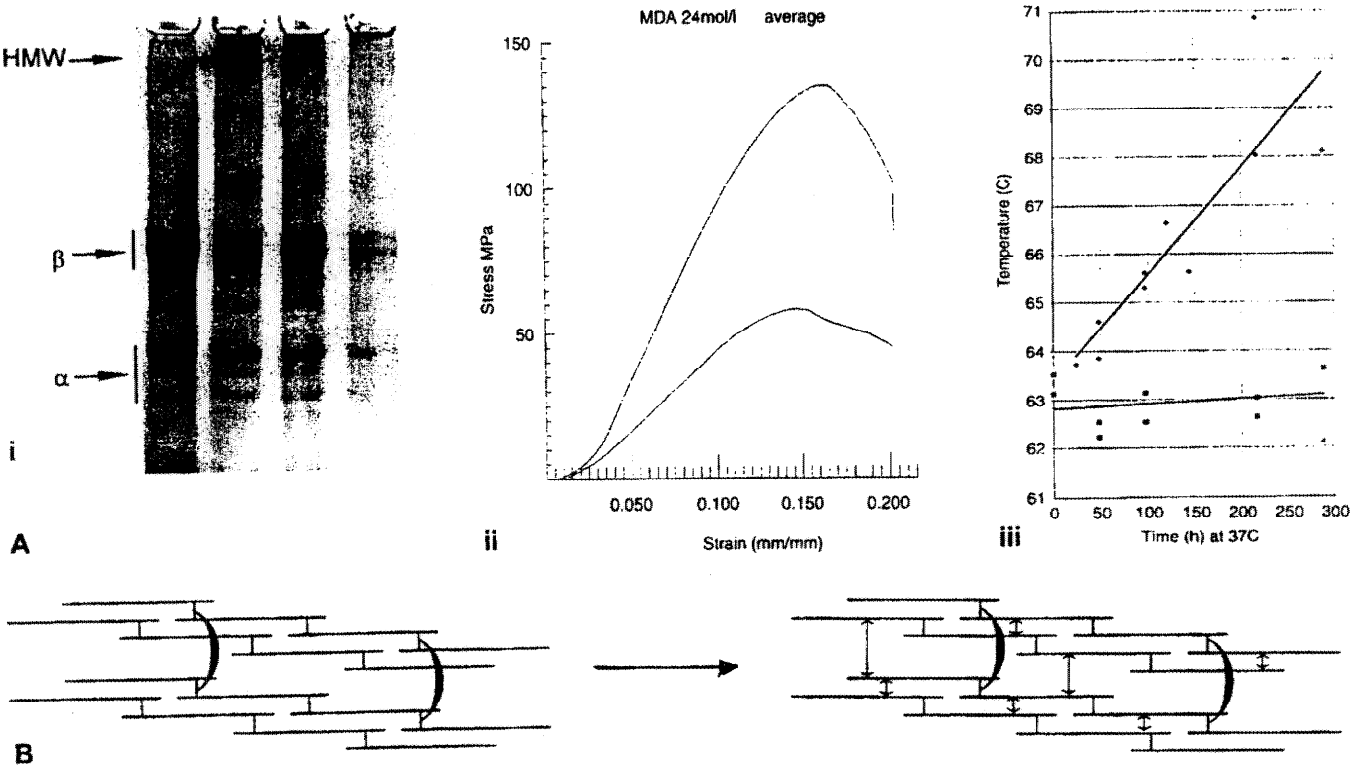

Fig. 4. **A** The effect of MDA cross-linking of collagen as assessed by (i) increasing molecular weight by SDS-PAGE; α and β represent single and dimeric collagen chains and HMW high molecular weight components. (ii) increased tensile strength as depicted by stress-strain curve after 24 h of incubation (iii) increasing thermal denaturation temperature with time of incubation by differential scanning calorimetry. **B** Schematic representation of the probable location of cross-links in the fibril (i) natural immature cross-links within the microfibre (**I**), natural mature inter fibril cross-links ()), (ii) additional MDA cross-links within and between microfibrils (↕)

cross-link. Studies to date suggest that MDA cross-links are labile and are destroyed by acid hydrolysis.

It has been shown that MDA will form cross-links such as LML when the pH is approximately five or less, but there is no convincing evidence that it occurs at the higher physiological pH, where NLMDD and β-LAA are favoured. It has, however, recently been suggested [39] that acidic conditions prevail in the immediate vicinity of the foam cell lesions seen in early atheroma. Such conditions could support the formation of the di-lysyl derivative.

Protein side-chain adducts. Non-cross-linking MDA adducts are also formed in the reaction of MDA with proteins. Malondialdehyde reacts with arginine side-chains to form NPO and this compound has been isolated from in vitro collagen-MDA incubations as it is reasonably resistant to acid [34]. Preliminary studies have identified its presence in skin of diabetic subjects and NPO could therefore be a potential biomarker [34].

It could be speculated that NPO reacts with lysine aldehyde present in collagen to form new cross-links. It is self-evident that the faster reaction of glycated proteins with MDA [16] must involve more than just lysine or arginine and possibly a reaction with pre-existing MDA adducts such as NPO.

Similarly, the MDA-lysine adduct, β-LAA, has been observed as a reduction product [40] and could be an epitope recognised by autoantibodies, as well as a target for elimination by macrophages [9].

The terminal α-amino-groups of proteins have also been shown to react with MDA in a manner similar to the ε-amino group of peptide bound lysine, for example, the valine N-terminus of haemoglobin [15].

Modification of cell-matrix interactions. The reactions with the side-chains of protein-bound lysine and arginine possibly not only affect the functions of collagen by making it less susceptible to degradative enzymes but could result in the formation of adducts that change the charge profile of the molecule and affect collagen-cell interactions. This effect would be particularly important if the particular arginine residues reacting with the MDA to form NPO are sites recognised by cell surface integrins, such as, the arginine-glycine-aspartic (RGD) sequences. Preliminary investigations have shown that MDA-collagen affects both the morphology and expression of the cells (unpublished observations). The response is similar to that reported for the reaction of glyoxal, which has been

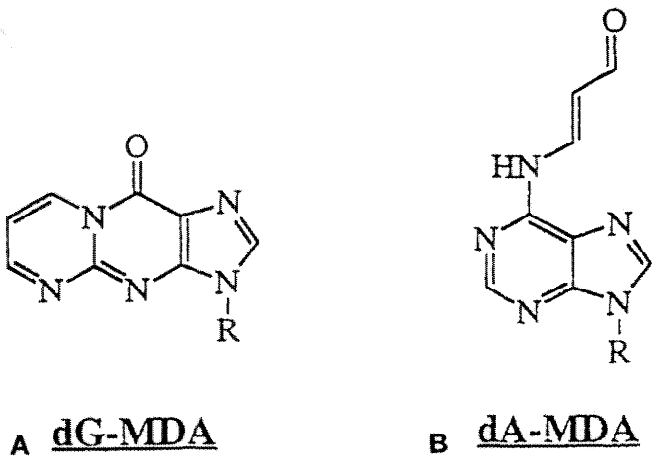

Fig. 5 A, B. Nucleotide adducts with Malondialdehyde. (**A**) Malondialdehyde-deoxy-guanosine adduct (pyrimido-(1,2)purine-10(3H)-one-2-deoxyribose) (**B**) Malondialdehyde-deoxy-adenosine adduct (*N*-6-oxypropenyl-2-deoxyribose)

shown to react fairly specifically with arginine in collagen to form imidazolones which block arginine containing integrin sites (e.g. arginine-glycine-aspartic) and lead to decreased adhesion and migration of cells [41].

MDA-nucleotide adducts

Several products of the reaction of nucleotides with MDA have been isolated and characterised (Fig. 5). Adding MDA to human cell cultures so that deoxyguanosine-MDA is three to six times normal has been shown to induce cell cycle arrest [42]. Two separate groups report isolation of deoxyguanosine-MDA from rodent livers [43,44]. All the non-cross-linking MDA adducts so far described disrupt base-pairing, and any MDA-DNA cross-link could easily cause the observed mutagenic and carcinogenic effects of MDA. Whatever the actual reactivity MDA has with DNA, repair systems must be present to recognise and replace it, as it has been shown that deoxyguanosine-MDA (Fig. 4) is present in urine [45].

Again, there is confusion due to investigations of the chemical reactions being carried out at a different pH. The fluorescence generated from MDA-DNA reactions at neutral pH has been attributed to DNA cross-linking but these studies were carried out at pH 5 and involved many processing steps before the final isolation of deoxycytosine-MDA-deoxyguanosine [46].

Incubation of the MDA analogue β-benzyloxyacrolein with calf thymus DNA at pH 6.5, has been reported to produce two adducts, deoxyguanosine-MDA and deoxyadenosine-MDA [47]. Chemically, MDA: guanosine adducts have been reported at ratios of 1:1 at pH 4.5 [48] and 2:1 at pH 7.0 [49],

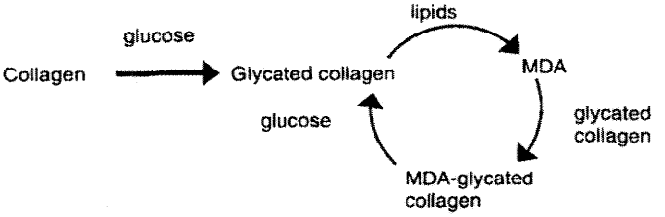

Fig. 6. Proposed schematic pathway of MDA cross-linking and side-chain modification of cardiovascular collagen in diabetes mellitus. The glucose glycated collagen stimulates oxidation of LDL producing MDA which then cross-links the collagen, stabilising the collagen and rendering it susceptible to further glycation and increased oxidation of the LDL and producing more MDA continuing a cycle of events

(Fig. 5) with analogous products for cytosine. Adducts of 3:1 have also been reported for MDA:adenine at pH 4.2 [50] (Fig. 5). Biological cross-links have, however, only been isolated using extensive borohydride reduction [46] which as described above has been questioned. The conclusion enforced is that there is almost certainly DNA-MDA cross-linking to some degree but no concrete data or consensus on its nature.

Concluding remarks

The inter-molecular cross-linking of collagen through MDA is important in the late complications of diabetes mellitus because it contributes to the stiffening of the cardiovascular tissue, although the nature of the cross-link remains to be determined. In addition it could be a link between glycation and further lipid peroxidation. The stabilisation of long-lived proteins such as collagen through MDA cross-linking not only reduces its optimal functioning but reduces its already low turnover and consequently allows further glycation by glucose and its oxidation products. This in turn increases the potential of the glycated collagen to initiate further oxidation of fatty acids releasing more MDA and thereby propagating a positive feedback cycle of protein and fatty acid damage (Fig. 6).

Changes in both physical properties and charge profile of the protein would be particularly important for the attachment of cells to the collagenous basement membrane. Recent studies have shown a pronounced change in the cell-matrix interactions after glycation of the matrix [41, 51]. This induces platelet aggregation, increasing the risk of thrombotic disease in the elderly and diabetic subjects.

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Two Distinct Pathways of Formation of 4-Hydroxynonenal

MECHANISMS OF NONENZYMATIC TRANSFORMATION OF THE 9- AND 13-HYDROPEROXIDES OF LINOLEIC ACID TO 4-HYDROXYALKENALS*

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The mechanism of formation of 4-hydroxy-2*E*-nonenal (4-HNE) has been a matter of debate since it was discovered as a major cytotoxic product of lipid peroxidation in 1980. Recent evidence points to 4-hydroperoxy-2*E*-nonenal (4-HPNE) as the immediate precursor of 4-HNE (Lee, S. H., and Blair, I. A. (2000) *Chem. Res. Toxicol.* 13, 698–702; Noordermeer, M. A., Feussner, I., Kolbe, A., Veldink, G. A., and Vliegenthart, J. F. G. (2000) *Biochem. Biophys. Res. Commun.* 277, 112–116), and a pathway via 9-hydroperoxylinoleic acid and 3*Z*-nonenal is recognized in plant extracts. Using the 9- and 13-hydroperoxides of linoleic acid as starting material, we find that two distinct mechanisms lead to the formation of 4-H(P)NE and the corresponding 4-hydro(pero)xyalkenal that retains the original carboxyl group (9-hydroperoxy-12-oxo-10*E*-dodecenoic acid). Chiral analysis revealed that 4-HPNE formed from 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HPODE) retains >90% *S* configuration, whereas it is nearly racemic from 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9*S*-HPODE). 9-Hydroperoxy-12-oxo-10*E*-dodecenoic acid is >90% *S* when derived from 9*S*-HPODE and almost racemic from 13*S*-HPODE. Through analysis of intermediates and products, we provide evidence that (i) allylic hydrogen abstraction at C-8 of 13*S*-HPODE leads to a 10,13-dihydroperoxide that undergoes cleavage between C-9 and C-10 to give 4*S*-HPNE, whereas direct Hock cleavage of the 13*S*-HPODE gives 12-oxo-9*Z*-dodecenoic acid, which oxygenates to racemic 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid; by contrast, (ii) 9*S*-HPODE cleaves directly to 3*Z*-nonenal as a precursor of racemic 4-HPNE, whereas allylic hydrogen abstraction at C-14 and oxygenation to a 9,12-dihydroperoxide leads to chiral 9*S*-hydroperoxy-12-oxo-10*E*-dodecenoic acid. Our results distinguish two major pathways to the formation of 4-HNE that should apply also to other fatty acid hydroperoxides. Slight (~10%) differences in the observed chiralities from those predicted in the above mechanisms suggest the existence of additional routes to the 4-hydroxyalkenals.

The complex processes of lipid peroxidation result in the formation of multiple products with the potential to interact and influence the outcome of normal cellular processes and control mechanisms. The pioneering work of Esterbauer and co-workers (1–3) on the production of cytotoxic molecules in peroxidation reactions led to the discovery of a group of conjugated aldehydes with toxic potential. Within this group, the most abundant member was identified as 4-hydroxy-2*E*-nonenal (4-HNE)[1]. In the ensuing years, 4-HNE has achieved status as one of the best recognized and most studied of the cytotoxic products of lipid peroxidation (4, 5). In addition to studies on its bioactivity, 4-HNE is commonly used as a biomarker for the occurrence and/or the extent of lipid peroxidation. The reviews on the production of 4-HNE include its involvement in cell cycle control (6), the oxidative alterations in Alzheimer's disease (7, 8), and its participation in the formation of etheno DNA-base adducts (9).

Despite the volumes of literature on the occurrence and activities of 4-HNE, there are comparatively few studies on how it is formed. It is recognized that linoleic acid and arachidonic acid are among the potential precursors for 4-HNE formation and that the nine carbons of 4-HNE are represented by the last nine carbons of these ω-6 essential fatty acids. It was also reported in the early work (4) that 15-hydroperoxy-eicosatetraenoic acid is a precursor. In 1990, Porter and Pryor (10) presented a hypothesis paper that proposed several mechanisms of 4-HNE formation involving the 4,5-epoxy derivative as the intermediate. The first experimental evidence for a pathway from fatty acid hydroperoxides to 4-HNE stemmed from the work of Gardner and Hamberg (11) on the biosynthesis of 4-HNE in broad bean extracts. They established that the aldehydic product of the reaction of 9-hydroperoxylinoleic acid with hydroperoxide lyase, namely 3*Z*-nonenal, can be converted to 4-hydroperoxy-2*E*-nonenal (4-HPNE) by a reaction of molecular oxygen, mainly catalyzed in this case by a 3*Z*-alkenal oxygenase. 4-HPNE is a simple reduction step removed from 4-HNE. Gardner and Hamberg (11) also substantiated an additional route to 4-HNE via peroxygenase reactions utilizing the co-substrates 3*Z*-nonenal and 4-HPNE; the existence of a nonenzymatic pathway was also implicated. Subsequent work by Gardner and Grove (12) showed that 3*Z*-nonenal is a substrate for soybean lipoxygenase, which thus could function as a 3*Z*-alkenal oxygenase and that the product is 4-HPNE. More recently, Noordermeer *et al.* (13) implicated nonenzymatic ox-

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[1] The abbreviations used are: 4-HNE, 4-hydroxy-2*E*-nonenal; 4-HPNE, 4-hydroperoxy-2*E*-nonenal; HPODE, hydroperoxy-octadecadienoic acid; 13*S*-HPODE, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; 9*S*-HPODE, 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid; SP, straight phase; HPLC, high pressure liquid chromatography; RP, reverse phase; GC, gas chromatography; MS, mass spectrometry; ESI, electrospray ionization; LC, liquid chromatography; MOX, methoxime; CD, circular dichroism.

ygenation of 3*Z*-alkenals (via 4-hydroperoxy intermediates) as the major pathway of production of 4-HNE and related 4-hydroxyalkenals in plant extracts. 4-HPNE also has been detected as a nonenzymatic breakdown product of 13-HPODE (14).

In the present work, we utilized both 9-HPODE and 13-HPODE as model fatty acid hydroperoxides to study the mechanisms of nonenzymatic formation of the 4-hydroxyalkenals. Both fatty acid hydroperoxides give rise to 4-H(P)NE, but each has differing rates and susceptibilities to inhibition by α-tocopherol. The use of chiral starting materials and analyses of stereochemistry of the products reveal a pathway from ω-6 hydroperoxides (13-HPODE) to the 4-hydroxyalkenals and insights into the stereochemistry of the nonenzymatic reactions.

EXPERIMENTAL PROCEDURES

Preparation of Hydroperoxides—13*S*-HPODE was synthesized from linoleic acid using soybean lipoxygenase (Sigma, Type V) and purified by preparative SP-HPLC (Alltech Econosil silica, 1.0 × 25 cm, hexane/isopropanol/acetic acid 100:1.5:0.1 by volume at 4 ml/min). 9*S*-HPODE was synthesized using a lipoxygenase preparation from tomato fruit (15) and purified using the same SP-HPLC conditions as above. The hydroperoxides were stored as a 5 mg/ml stock solution in acetonitrile or methanol under argon at −80 °C.

Autoxidation Reactions—25-μg aliquots of the linoleic acid hydroperoxides were transferred into 1.5-ml plastic tubes and evaporated from the solvent under a stream of nitrogen. In some experiments, α-tocopherol from a stock solution in ethanol, 5 or 10% (w/w), was added prior to evaporation. The tubes were placed in a 37 °C oven and removed after a 1-, 2-, or 4-h incubation. 30 μl of column solvent was added, and the complete content was injected on an RP-HPLC column (Waters Symmetry C18 5 μm, 0.46 × 25 cm) eluted with a solvent of acetonitrile/water/acetic acid (60:40:0.01 by volume) at a flow rate of 1 ml/min. The column effluent was monitored using an HP 1040A diode array detector. For product identification and chiral analyses, 5-mg aliquots of 13*S*- and 9*S*-HPODE were autoxidized for 5 h at 37 °C.

Identification of Autoxidation Products—4-HPNE was prepared from an incubation of 1 mg of 9*S*-HPODE with a crude bacterial lysate of a hydroperoxide lyase from melon fruit expressed in *Escherichia coli* (16). 4-HPNE (retention time 5.7 min) was isolated using a Waters Symmetry C18 5-μm column (0.46 × 25 cm) eluted with a solvent of acetonitrile/water/acetic acid (60:40:0.01) at a flow rate of 1 ml/min. The collected product was evaporated from acetonitrile, extracted using a 100-mg C18 cartridge (Varian) eluted with diethyl ether, and dried over Na_2SO_4. An aliquot of the 4-HPNE was reduced with triphenylphosphine to 4-HNE, repurified by RP-HPLC (retention time 4.6 min), and derivatized with bis(trimethylsilyl)trifluoroacetamide to the trimethylsilyl ether derivative. GC-MS analysis yielded the following diagnostic fragments: *m/z* 199 [M^+ − CHO]; *m/z* 157 [CHO–C_2H_2–CH–$OSi(CH_3)_3^+$]; and *m/z* 129 [CHO–C_2H_2–CH–$OSi(CH_3)_3^+$ − CO]. ^1H NMR spectra of 4-HPNE were recorded in CD_3CN on a Bruker WM 400-MHz spectrometer using residual CH_3CN as an internal reference (δ = 1.92 ppm): 9.58 ppm, d, J = 7.8 Hz, H1; 6.9 ppm, dd, J = 15.9 Hz, 6.2 Hz, H3; 6.25 ppm, ddd, J = 15.9 Hz, 7.8 Hz, 1.2 Hz, H2; 4.6 ppm, q, J ~ 6.5 Hz, H4.

9-Hydroperoxy-12-oxo-10*E*-dodecenoic acid was prepared from a 5-mg reaction of 13*S*-HPODE with an expressed and purified recombinant hydroperoxide lyase from melon fruit in 25 ml of 50 mM Tris-HCl, pH 7.5 (16). After 5 min, the reaction was terminated by adding 1 N HCl up to pH 4.5 and extracted twice with 30 ml of ethyl acetate containing 250 μg α-tocopherol. The pooled organic phases were washed with water, dried over Na_2SO_4, and evaporated under reduced pressure. The crude mixture was kept under an atmosphere of oxygen at 37 °C for 7 days. 9-Hydroxy-12-oxo-10*E*-dodecenoic acid and 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid were isolated by RP-HPLC using a Beckman Ultrasphere ODS column (1.0 × 25 cm) eluted with acetonitrile/water/acetic acid (37.5:62.5:0.01 by volume) at 4 ml/min (retention times of 5.4 and 7.3 min, respectively). The collected fractions were evaporated from acetonitrile, and the products were extracted using a 100-mg C18 cartridge (Varian) eluted with ethyl acetate and dried over Na_2SO_4. For the GC-MS analysis, 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid was reduced with triphenylphosphine, treated with methoxime hydrochloride and ethereal diazomethane, and purified by RP-HPLC. The *syn*- and *anti*-isomers gave essentially the same fragment ions at *m/z* 240 [M^+ − OCH_3], 114 [$COC_2H_2CH{=}NOCH_3$]$^+$, and 86 [$C_2H_2CH{=}NOCH_3$]$^+$. ^1H NMR spectra were recorded in $CDCl_3$ on a Bruker WM 400 MHz spectrometer using residual $CHCl_3$ as internal reference (δ = 7.26 ppm). 9-Hydroperoxy-12-oxo-10*E*-dodecenoic acid: 9.61 ppm, d, J = 7.7 Hz, H12; 6.79 ppm, dd, J = 15.9 Hz, 6.3 Hz, H10; 6.30 ppm, ddd, J = 15.9 Hz, 7.7/7.8 Hz, 1.0 Hz, H11; 4.65 ppm, dt, J = 6.2 Hz, 0.9 Hz, H9; 2.36 ppm, t, J = 7.4 Hz, H2. 9-Hydroxy-12-oxo-10*E*-dodecenoic acid: 9.59 ppm, d, J = 7.8 Hz, H12; 6.82 ppm, dd, J = 15.7 Hz, 4.7 Hz, H10; 6.31 ppm, ddd, J = 15.7 Hz, 7.8 Hz, 1.4 Hz, H11; 4.43 ppm, m, 1H, H9.

The 8,13-diHPODEs and 9,14-diHPODEs were isolated from a 5-mg autoxidation of 13*S*-HPODE or 9*S*-HPODE, respectively, by RP-HPLC (Beckman Ultrasphere ODS 10 μm, 1.0 × 25 cm eluted with a solvent of acetonitrile/water/acetic acid (50:50:0.01) at a flow rate of 4 ml/min). For ^1H NMR analysis, the collected 8,13-diHPODEs were reduced with $NaBH_4$, methylated, and further purified by SP-HPLC using a Whatman Partisil 5-μm column (0.46 × 25 cm) and a solvent of hexane/isopropanol/acetic acid (90:10:0.1 by volume) at a flow rate of 1 ml/min. The ^1H NMR spectra were recorded in C_6D_6 using residual benzene as internal reference (δ = 7.24 ppm). Aliquots of the samples collected from the initial RP-HPLC separation were used for analysis by LC-ESI-MS. LC-coordination ion spray-MS of the Ag^+ adduct ions of the linoleic acid dihydroperoxides was performed on a triple-stage quadrupole TSQ7000 instrument (Finnigan, San Jose, CA) using conditions essentially as described (17). The HPLC parameters were: Beckman Ultrasphere Si column (0.2 × 25 cm) eluted with hexane/isopropanol/acetic acid (90:10:0.1 by volume) at a flow rate of 0.15 ml/min. A solution of $AgBF_4$ in isopropanol (0.3 mM) was mixed to the column effluent before the ESI interface using a syringe pump at a pump rate of 75 μl/min. For the GC-MS analysis the pairs of diastereomers were collected from RP-HPLC, reduced with triphenylphosphine, methylated using ethereal diazomethane, and further purified by SP-HPLC using a Beckman Ultrasphere Si column (0.46 × 25 cm) eluted with hexane/isopropanol/acetic acid (90:10:0.1 by volume) at 1 ml/min. The collected products were hydrogenated using 5% palladium on alumina and treated with bis(trimethylsilyl)trifluoroacetamide/pyridine. GC-MS was performed on a Finnigan Incos 50 mass spectrometer connected to an HP5890A gas chromatograph. For GC, an 8-m OV1701 column was used with a temperature program starting at 150 °C (1 min isotherm) and a rate of 15 °C/min to 300 °C (4 min isotherm).

Quantification of 4-HPNE and 9-Hydroperxy-12-oxo-10E-dodecenoic acid—4-HPNE and 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid were quantified using an external calibration curve obtained by injecting aliquots of 4-HNE (5–100 ng) on the RP-HPLC system used for product analysis and plotting against peak height.

Chiral Resolution of 4-H(P)NE and 9-Hydro(pero)xy-12-oxo-10E-dodecenoic Acid—100 μg of a racemic standard of 4-HNE (Cayman Chemical, Ann Arbor, MI) were reacted with a molar excess of methyl oxime hydrochloride in 20 μl of pyridine at room temperature overnight. The solvent was evaporated, and the residue was dissolved in 1 ml of methylene chloride and washed three times with 500 μl of water to remove residual reagent and pyridine. The 4-HNE methoxime derivatives were separated on a Waters Symmetry C18 5-μm column (0.46 × 25 cm) eluted with a solvent of acetonitrile/water/acetic acid (50:50:0.01 by volume) at a flow rate of 1 ml/min and UV detection at 235 nm. The two isomers (*syn* and *anti*) eluted at 10.4 and 11.2 min retention time, respectively. The later eluting isomer was analyzed by chiral phase HPLC using a Chiralpak AD (0.46 × 25 cm) column eluted with hexane/ethanol (90:10 by volume) at a flow rate of 1 ml/min and monitored using an HP 1040A diode array detector.

20 μg of the chemically synthesized 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid were reduced with triphenylphosphine and treated with methyl oxime hydrochloride in pyridine overnight. The sample was extracted, washed, evaporated, and dissolved in 20 μl of methanol. To this solution a few drops of ethereal diazomethane were added, and the sample was evaporated immediately. The *syn*- and *anti*-methoxime (MOX) isomers (retention times of 10.0 and 10.8 min, respectively) were isolated from RP-HPLC (Waters Symmetry C18 5-μm column 0.46 × 25 cm) using acetonitrile/water/acetic acid (37.5:62.5:0.01 by volume) as solvent at a flow rate of 1 ml/min. Chiral analysis of the earlier eluting isomer was performed using the chiral phase HPLC conditions described above.

From a 5-mg autoxidation of 13*S*-HPODE and 9*S*-HPODE (5 h at 37 °C), 4-HPNE and 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid were isolated by RP-HPLC using a Beckman Ultrasphere ODS 10-μm column (1.0 × 25 cm) eluted with a solvent of acetonitrile/water/acetic acid (50:50:0.01) at a flow rate of 4 ml/min. The products were reduced with an excess of triphenylphosphine and then further derivatized, purified, and analyzed essentially as described for the racemic standards.

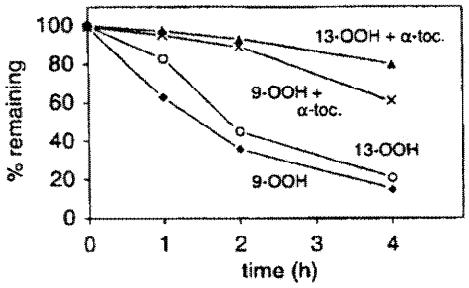


FIG. 1. **Time course of the degradation of linoleic acid hydroperoxides in the presence and absence of α-tocopherol.** 25-μg aliquots of the hydroperoxides were autoxidized with or without 5% (w/w) α-tocopherol (*α-toc.*) at 37 °C and analyzed as described under "Experimental Procedures." The remaining HPODEs are expressed as a percentage of the starting amount (t = 0, set at 100%).

CD Spectroscopy—The enantiomers of the racemic 4-HNE methoxime derivative and methyl-9-hydroxy-12-oxo-10*E*-dodecenoic acid methoxime derivative were collected from the chiral phase HPLC separations. The four products were evaporated from solvent under a stream of nitrogen and dissolved in 50 μl of dry acetonitrile. 1 μl of 1,8-diazabicyclo[5,4,0]undec-7-ene and a few grains of 1-(2-naphthoyl)imidazole (Fluka) were added. The reaction was kept at room temperature overnight, and the solvent was evaporated. The residue was dissolved in 1 ml of methylene chloride, washed three times with water, and evaporated; finally the naphthoate derivatives were purified by SP-HPLC using a Beckman Ultrasphere Si column (0.46 × 25 cm) eluted with hexane/isopropanol/acetic acid (100:1:0.1 by volume) at a flow rate of 1 ml/min. For UV and CD spectroscopy, the collected products were evaporated from column solvent and dissolved in acetonitrile to a final $A_{239\ nm}$ of 1 absorbance unit (4-HNE derivatives) or 0.2 absorbance units (methyl-9-hydroxy-12-oxo-10*E*-dodecenoic acid derivatives). CD spectra were recorded on a JASCO J-700 spectropolarimeter.

RESULTS

Time Course of Degradation of Linoleic Acid Hydroperoxides—The 13- and 9-linoleic acid hydroperoxides were autoxidized in 25-μg aliquots as a dry film in open 1.5-ml plastic tubes at 37 °C for 1, 2, or 4 h. At each time point column solvent was added to the tubes, and the complete sample was injected on RP-HPLC. The time course of the decay of 9- and 13-HPODE in the presence and absence of α-tocopherol is shown in Fig. 1. Over the course of 4 h at 37 °C the plain hydroperoxides are about 90% degraded, whereas in the presence of α-tocopherol the degradation is slowed down to about 70–80% remaining hydroperoxides after 4 h.

RP-HPLC Analysis of Autoxidation Reactions—The polar products formed in the autoxidation reactions were analyzed by RP-HPLC (Fig. 2). In these chromatograms, the autoxidations of 13*S*-HPODE (Fig. 2*A*) and 9*S*-HPODE (Fig. 2*B*) were analyzed after 1 h, and the autoxidation of 13*S*-HPODE in the presence of 5% (w/w) α-tocopherol was analyzed after 4 h (Fig. 2*C*). The polar products with distinctive UV chromophores were designated as 1–7. Compounds 1 and 3 were formed from both hydroperoxides. Compounds 2, 4, and 5 were products of the 13-hydroperoxide, and 6 and 7 were products from 9-HPODE. The *arrows* in Fig. 2 indicate the retention time of 4-HNE, detected only as a minor product from 13*S*- and 9*S*-HPODE in these experiments (<5 ng/25 μg HPODE). Over the course of the 4-h period of autoxidation, no additional abundant products were formed, and there were only minor changes in the product pattern.

Identification of Products—Compound 3 was identified as 4-HPNE based on identical UV spectra (λ_{max} 223 nm in RP-HPLC column solvent; Fig. 3) and co-chromatography with a synthesized standard. Furthermore, treatment of compound 3 with triphenylphosphine yielded a product that co-chromatographed on RP-HPLC with authentic 4-HNE.

The UV spectra of compounds 1 and 3 (4-HPNE) were almost

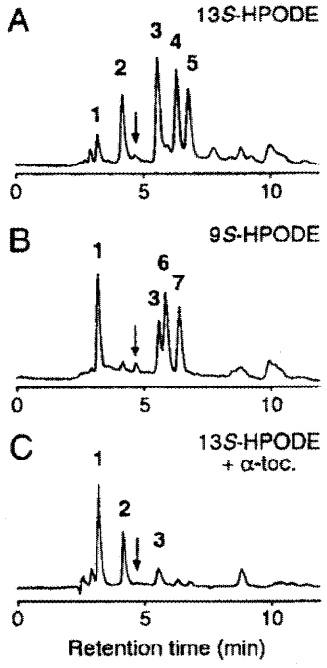


FIG. 2. **RP-HPLC analysis of the autoxidation reactions of linoleic acid hydroperoxides.** *A*, autoxidation of 13*S*-HPODE. *B*, autoxidation of 9*S*-HPODE. *C*, autoxidation of 13*S*-HPODE in the presence of 5% (w/w) α-tocopherol (*α-toc.*). 25-μg aliquots of the hydroperoxides were auto-oxidized at 37 °C for 1 h (*A* and *B*) or 4 h (*C*), and the complete reaction mixture was analyzed on RP-HPLC (Waters Symmetry C18 5-μm, 0.46 × 25 cm, acetonitrile/water/acetic acid 60:40:0.01 by volume, at a flow rate of 1 ml/min). The column effluent was monitored using an HP 1040A diode array detector. The chromatograms shown were recorded at 220 nm (full scale absorbance, 25 milliabsorbance units).

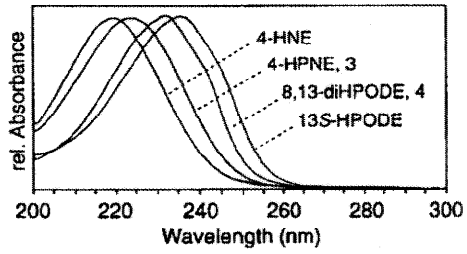


FIG. 3. **UV spectra of 4-HNE, compound 3 (4-HPNE), compound 4 (8,13-diHPODE), and 13S-HPODE.** The UV spectra were recorded in column solvent (acetonitrile/water/acetic acid 60:40:0.01 by volume) using an HP 1040A diode array detector. The spectra are normalized to λ_{max}.

indistinguishable, but compound 1 eluted at a much earlier retention time. The reduction of compound 1 with triphenylphosphine resulted in a slightly more polar product on RP-HPLC and with a UV spectrum almost identical to 4-HNE (Fig. 3). Based on the chromatographic and spectroscopic data, compound 1 was suspected to be 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid, a C-12 aldehyde derivative that retains the original carboxyl group of the starting fatty acid hydroperoxide. An authentic standard of 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid was synthesized through the following steps: (i) preparation of 13*S*-HPODE from linoleic acid using soybean lipoxygenase, (ii) cleavage of the hydroperoxide using a recombinant hydroperoxide lyase from melon fruit (16), (iii) autoxidation of the 12-oxo-9*Z*-dodecenoic acid cleavage product in the presence of α-tocopherol, and (iv) isolation of the 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid by RP-HPLC. The identification of compound 3 as 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid was confirmed by ^{1}H NMR and by GC-MS

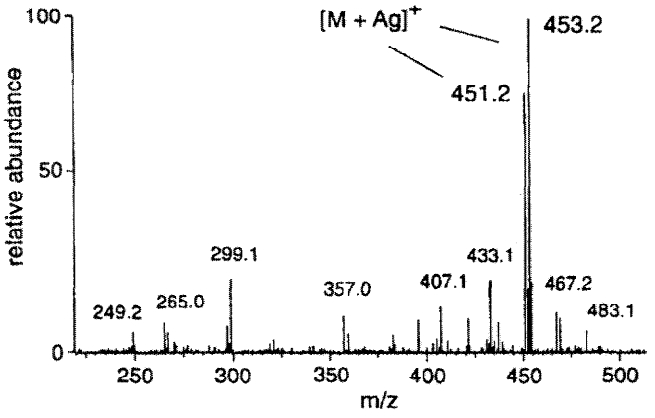


FIG. 4. **LC-coordination ion spray (Ag^+) mass spectrum of 8,13-dihydroperoxy-9*E*,11*E*-octadecadienoic acid (compound 5).** Compound 5 was isolated from the autoxidation of 5 mg of 13*S*-HPODE by RP-HPLC and analyzed by LC-CSI-MS with $AgBF_4$ using the conditions described under "Experimental Procedures."

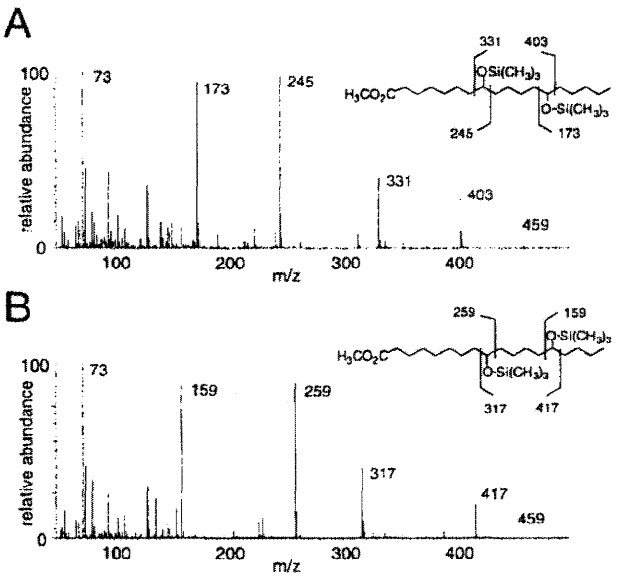


FIG. 5. **Mass spectra (electron impact mode) of 8,13-dihydroperoxy-9*E*,11*E*-octadecadienoic acid (*A*) and 9,14-dihydroperoxy-10*E*,12*E*-octadecadienoic acid (*B*) of the reduced, methylated, and hydrogenated trimethylsilyl ether derivative.** *A*, mass spectrum of compound 5 isolated from the autoxidation of 13*S*-HPODE after reduction, hydrogenation, and derivatization with diazomethane and bis(trimethylsilyl)trifluoroacetamide. *B*, mass spectrum of compound 7 isolated from the autoxidation of 9*S*-HPODE after similar derivatization.

analysis of the triphenylphosphine-reduced methyl ester methoxime derivative.

Compound 2 was identified by LC-MS and ^1H NMR as 11-oxo-9*Z*-undecenoic acid (data not shown). This product is not directly involved in the pathways leading to the formation of the 4-hydro(pero)xyalkenals. Further characterization of this product and its mechanism of formation will be reported elsewhere.

As determined by the RP-HPLC analysis, compounds 4 and 5 (derived from 13*S*-HPODE; Fig. 2*A*) and 6 and 7 (derived from 9*S*-HPODE, Fig. 2*B*) were formed consistently in the same relative amount to each other during the 4-h time period of autoxidation. They showed identical UV spectra indicative of a *trans,trans* conjugated diene (λ_{max} 231 nm; Fig. 3) (18), giving the strong implication that these products were pairs of diastereomers. LC-ESI-MS analysis of the Ag^+-adduct ion revealed two [M + Ag^+] adduct ions at *m/z* 451 and 453 for compounds 4, 5, 6, and 7 (Fig. 4). This corresponds to a molecular weight of 344, which is compatible with linoleic acid dihydroperoxides. To reveal the position of the hydroperoxide groups on the fatty acid carbon chain, GC-MS analysis (electron impact mode) was performed on the reduced, methylated, and hydrogenated bis-trimethylsilyl-ether derivatives. The mass spectra of derivatized compounds 4 and 5 (derived from 13*S*-HPODE) showed characteristic ions at *m/z* 459 [M − CH_3]$^+$, 245 [$CH_3CO_2C_8H_{13}OSi(CH_3)_3$]$^+$ and 331 [$HCOSi(CH_3)_3C_5H_9OSi(CH_3)_3C_5H_{11}$]$^+$ (indicating the C-8 hydroxyl), and 403 [$CH_3CO_2C_8H_{13}OSi(CH_3)_3C_5H_9OSi(CH_3)_3$]$^+$ and 173 [$HCOSi(CH_3)_3C_5H_{11}$]$^+$ (indicating the C-13 hydroxyl) (Fig. 5*A*). Finally, ^1H NMR of the reduced compounds 4 and 5 (8,13-dihydroxyoctadecadienoates) fully supported the structures. ^1H NMR (400 MHz, in deuterated benzene using 7.24 ppm for the residual protons in the solvent) gave for the methyl ester of the 8,13-dihydroxyoctadecadienoate derivative of compound 4: δ (ppm) 0.94, t, 3 protons, H18; 0.98 (d, two protons, –O*H*, $J_{8,\text{-OH}} = J_{13,\text{-OH}} = 3.5$ Hz); 1.2–1.8 (m, 18 protons, H3-H7, and H14-H17); 2.14 (t, 2 protons, H2); 4.00 (m, 2 protons, H8, H13); 5.67 (m, 2 protons, H9, H12); and 6.24 (m, 2 protons, H10, H11). These data confirm the symmetry of the 1,6-dihydroxy-2,4-diene system; the olefinic region shows two complex multiplets, each comprised of two superimposed protons (H10/H11 at 6.24 ppm and H9/H10 at 5.67 ppm), and similarly a superimposed signal for the two geminal hydroxy protons (H8, H13 at 4.00 ppm). The fact that these signals consist of overlapping pairs of protons of identical chemical shift results in nonlinear effects that precluded a ready assignment of the coupling constant across the double bonds. For example, decoupling of the signal for H9/H12 at 5.67 ppm caused the downfield signal for the internal pair of olefinic protons to simplify to a singlet (rather than a doublet), a predictable result based on the lack of coupling between the two superimposed proton signals for H10/H11 (19). Despite their complexity, the ^1H NMR spectra were entirely supportive of the structures deduced from the UV and MS data.

Thus, analysis by LC-MS, GC-MS, UV spectroscopy, and ^1H NMR identified compounds 4 and 5 as 8,13-dihydroperoxyoctadeca-9*E*,11*E*-dienoic acids, presumably a pair of diastereomers with the 8*R*,13*S* and 8*S*,13*S* configurations. GC-MS analysis of the equivalent derivatives of compounds 6 and 7 derived from 9*S*-HPODE showed major fragments at *m/z* 459 [M − CH_3]$^+$, 259 [$CH_3CO_2C_9H_{15}OSi(CH_3)_3$]$^+$ and 317 [$HCOSi(CH_3)_3C_5H_9OSi(CH_3)_3C_4H_9$]$^+$ (indicating the C-9 hydroxyl), and 417 [$CH_3CO_2C_9H_{15}OSi(CH_3)_3C_5H_9OSi(CH_3)_3$]$^+$ and 159 [$HCOSi(CH_3)_3C_4H_9$]$^+$ (indicating the C-14 hydroxyl) (Fig. 5*B*). Compounds 6 and 7 were thus identified as 9,14-dihydroperoxyoctadeca-10*E*,12*E*-dienoic acids, also presumably a pair of diastereomers with the 9*S*,14*S* and 9*S*,14*R* configuration.

Time Course of Formation of 4-HPNE and 9-Hydroperoxy-12-oxo-10E-dodecenoic Acid—In Fig. 6 the time course of the formation of 4-HPNE (compound 3) and 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid (compound 1) during the autoxidation of 13*S*- and 9*S*-HPODE is shown. Starting with 25 μg of 13*S*-HPODE, ~100 ng of 4-HPNE are detected after 4 h of autoxidation, whereas from the same amount of 9*S*-HPODE, less than half as much 4-HPNE is detected (Fig. 6*A*). In the formation of 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid, more is generated from 9*S*-HPODE (~100 ng from 25 μg) than from 13*S*-HPODE (~30 ng from 25 μg) (Fig. 6*B*).

Autoxidation of 13S-HPODE in the Presence of α-Tocopherol—Autoxidations of 25-μg aliquots of 13*S*-HPODE as a dry film were carried out in the presence of 5% α-tocopherol for 1, 2, and 4 h. As shown in Fig. 1, the rate of decay of the

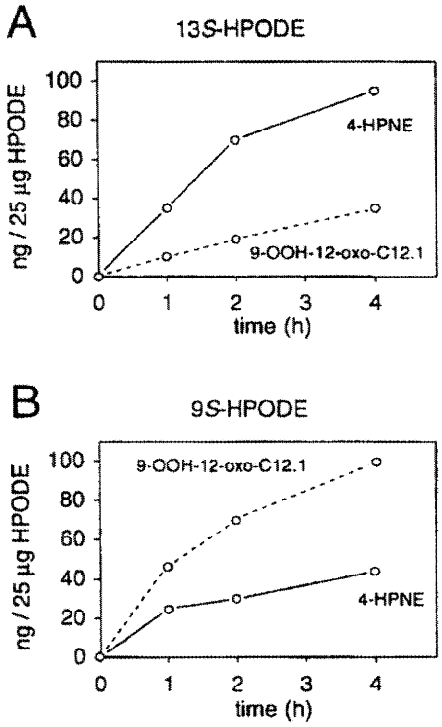


FIG. 6. **Time course of the formation of 4-HPNE and 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid from 13*S*-HPODE (*A*) and 9*S*-HPODE (*B*).** 25-µg aliquots of the hydroperoxides were autoxidized at 37 °C for the time indicated and analyzed as described under "Experimental Procedures." 4-HPNE and 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid were quantified based on external calibration using 4-HNE as a standard.

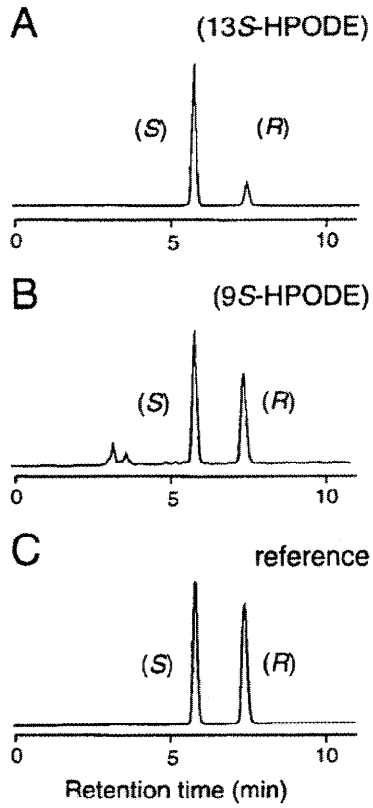


FIG. 7. **Chiral phase HPLC analysis of 4-H(P)NE (methoxime derivative) derived from 13*S*-HPODE (*A*) and 9*S*-HPODE (*B*).** 4-HPNE isolated from autoxidations was reduced with triphenylphosphine and derivatized and purified by RP-HPLC as described under "Experimental Procedures." *A*, analysis of 4-HPNE isolated from the autoxidation of 5 mg of 13*S*-HPODE. *B*, analysis of 4-HPNE isolated from the autoxidation of 5 mg of 9*S*-HPODE. *C*, racemic reference of 4-HNE. Chiral resolution was performed on a Chiralpak AD column (0.46 × 25 cm) eluted with hexane/ethanol (90:10 by volume) at a flow rate of 1 ml/min and UV detection at 235 nm. The elution order was determined by CD spectroscopy of the collected enantiomers.

hydroperoxide is decreased in the presence of α-tocopherol. A representative chromatogram obtained after 4-h autoxidation at 37 °C shows the formation of compound 1 (9-hydroperoxy-12-oxo-10*E*-dodecenoic acid) as the major product with absorbance at 220 nm (Fig. 2*C*). 4-HPNE (compound 3) and the UV-absorbing conjugated diHPODEs are only minor products in these experiments.

Chiral Analysis of 4-HPNE—To provide insights into the mechanism(s) of formation of 4-HPNE, an HPLC method for the chiral resolution of the more stable reduction product 4-HNE was developed. Injection of underivatized 4-HNE on the chiral column used resulted in the reaction of 4-HNE with the chiral stationary phase (Chiralpak AD). Therefore, the aldehyde group was derivatized to the MOX derivative. The resulting *syn*- and *anti*-isomers were readily resolved using RP-HPLC. When the later eluting oxime isomer from the RP-HPLC separation was injected on chiral phase HPLC, the enantiomers in a commercial standard of 4-HNE were widely resolved with retention times of 5.7 and 7.4 min (Fig. 7*C*). The earlier eluting MOX isomer was also resolved into two enantiomers, but with less resolution. The elution order of the enantiomers from the chiral phase HPLC separation was determined using CD spectroscopy (see below).

From separate 5-mg autoxidations of 13*S*- and 9*S*-HPODE, the 4-HPNE product was isolated by RP-HPLC, reduced with triphenylphosphine, and converted to the MOX derivative. The *syn*- and *anti*-isomers of the MOX derivative were resolved on RP-HPLC as described above for the racemic standards. Fig. 7*A* shows the chiral phase HPLC elution profile of the later eluting MOX isomer of 4-HNE derived from 13*S*-HPODE. Integration of the peak areas of the enantiomers gave a 90:10 ratio of 4*S*-HNE to 4*R*-HNE. Similar chiral analysis of the 4-HPNE product from a 9*S*-HPODE auto-oxidation showed that it was formed as a virtually racemic mixture (54% *S* and 46% *R*; Fig. 7*B*).

Chiral Analysis of 9-Hydroperoxy-12-oxo-10E-dodecenoic Acid—A method for chiral analysis of 9-hydro(pero)xy-12-oxo-10*E*-dodecenoic acid was developed for the methyl ester derivative along the same lines as for 4-HNE. Injection of the earlier eluting methyl ester MOX isomer from RP-HPLC resulted in the resolution into two enantiomers as shown in Fig. 8*C* (retention times of 9.4 and 12.9 min). When the 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid derived from autoxidation of 5 mg of 13*S*-HPODE was analyzed using this method, it was found to be an almost racemic mixture (53:47 *S* to *R*) (Fig. 8*A*). In contrast, 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid was formed from 9*S*-HPODE in an *S*/*R* enantiomer ratio of 91:9 (Fig. 8*B*).

CD Spectroscopy—The absolute configuration of the enantiomers of the MOX-derivatized 4-HNE and 9-hydroxy-12-oxo-10*E*-dodecenoic acid was determined by CD spectroscopy to determine the elution order from the chiral phase HPLC separations (20). The enantiomers of both products were collected from chiral phase HPLC, and the hydroxy group was derivatized with 2-naphthoyl-imidazole to introduce a second chromophore at the chiral center as depicted in Fig. 9. The exciton-coupled circular dichroism method of CD spectroscopy uses the interaction of two chromophores at the chiral center to define the absolute configuration. To delineate the absolute configuration of a particular chiral molecule from the CD spectrum,

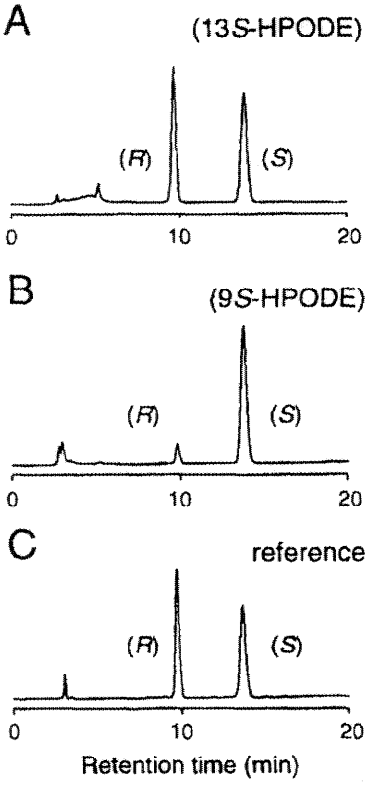


FIG. 8. **Chiral phase HPLC analysis of 9-hydroxy-12-oxo-10*E*-dodecenoic acid methyl ester (methoxime derivative) derived from 13*S*-HPODE (*A*) and 9*S*-HPODE (*B*).** 9-Hydroperoxy-12-oxo-10*E*-dodecenoic acid isolated from autoxidations was reduced with triphenylphosphine and derivatized and purified by RP-HPLC as described under "Experimental Procedures." *A*, analysis of 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid isolated from the autoxidation of 5 mg of 13*S*-HPODE. *B*, analysis of 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid isolated from the autoxidation of 5 mg of 9*S*-HPODE. *C*, racemic reference of 9-hydroxy-12-oxo-10*E*-dodecenoic acid. Chiral resolution was performed on a Chiralpak AD column (0.46 × 25 cm) eluted with hexane/ethanol (90:10 by volume) at a flow rate of 1 ml/min and UV detection at 235 nm. The elution order was determined by CD spectroscopy of the collected enantiomers.

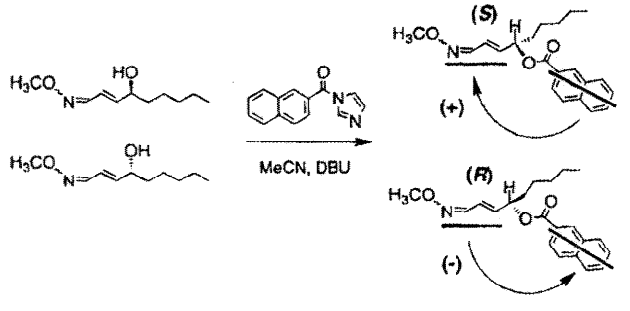


FIG. 9. **Derivatization of the 4-HNE methoxime derivative to the 2-naphthoate.** The chromophoric derivatives for CD spectroscopy were synthesized by derivatization of the enantiomers of methoxime 4-HNE, collected from the chiral phase separation, with 1-(2-naphthoyl)imidazole. The sign of the Cotton effects of the two enantiomers can be predicted from the left- (−) and right-handed (+) sense between the transition moments (*thick black line*) of the chromophores as indicated by the *curved arrows*. In this case, the *S* enantiomer of the derivatized 4-HNE has positive chirality.

the molecule is represented in the Newman projection. If the chirality of the electric transition moments of the first to the second chromophore is clockwise, defined as positive, the CD shows a positive first and a negative second Cotton effect; if the chirality is counter clockwise, defined as negative, the CD

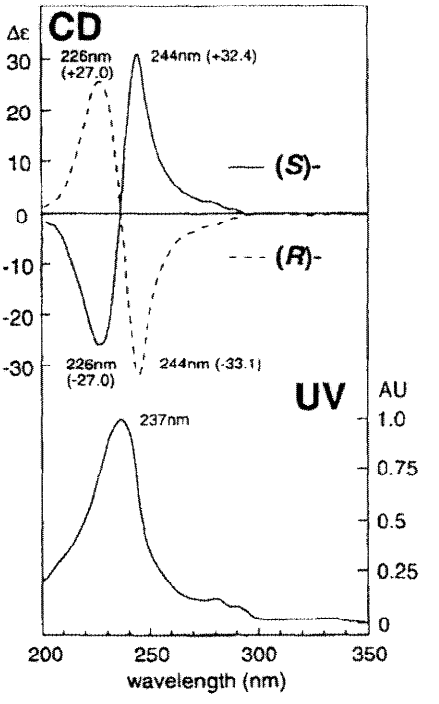
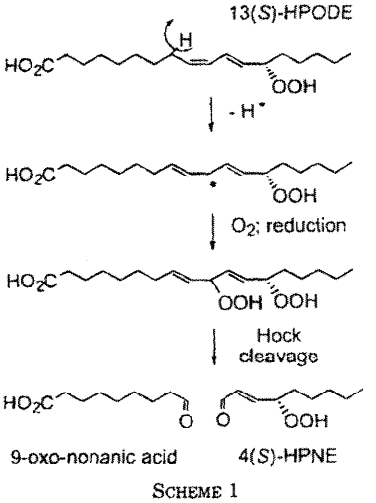


FIG. 10. **CD and UV spectra of *R*- and *S*-4-HNE (2-naphthoate, methoxime derivative) in acetonitrile.** The enantiomers of the methoxime derivative of 4-HNE were resolved using chiral phase HPLC and further derivatized to the 2-naphthoates (Fig. 9 and "Experimental Procedures"). The CD spectrum of the first eluting enantiomer from the chiral phase separation of methoxime 4-HNE has a positive first and a negative second Cotton effect (*solid line*); therefore it has positive chirality (*S* configuration; Fig. 9) (20).

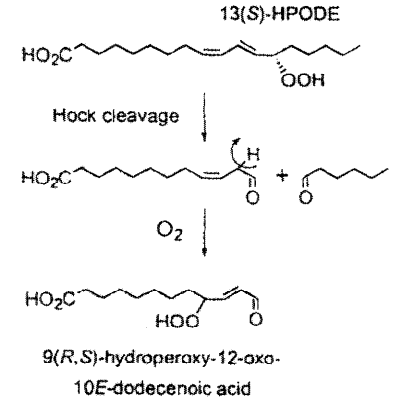


SCHEME 1

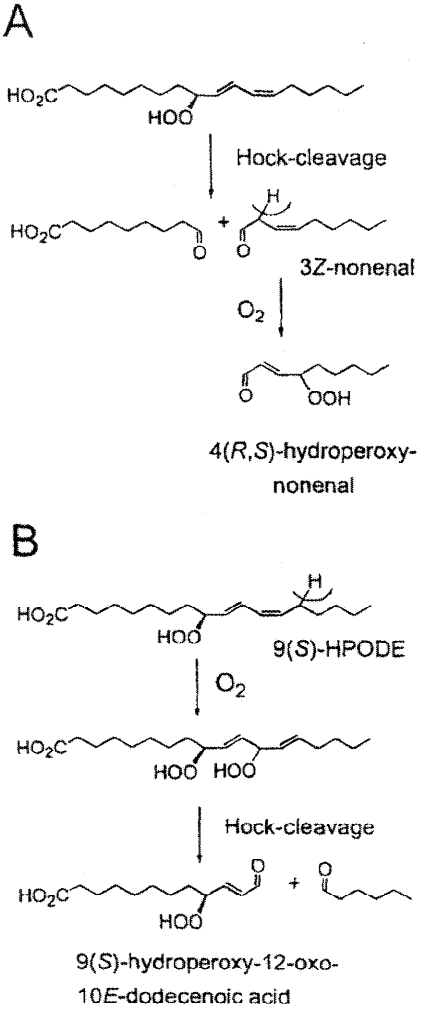
shows a negative first and a positive second Cotton effect.

The 2-naphthoate derivative of the first eluting enantiomer of the MOX-derivatized 4-HNE from the chiral column resolution showed a positive first Cotton effect at 244 nm ($\Delta\epsilon$, +32.4) and a negative second Cotton effect at 226 nm ($\Delta\epsilon$, −27.0) (Fig. 10). Thus, the CD spectrum of this enantiomer has a clockwise (positive) sense between the transition moments of the two chromophores as depicted in the projection in Fig. 9; therefore its absolute configuration is *S*. The later eluting enantiomer exhibited a mirror-image CD spectrum with extrema at 244 nm ($\Delta\epsilon$, −33.1) and 226 nm ($\Delta\epsilon$, +27.0), resulting in a negative chirality; the absolute configuration is *R* (Fig. 10).

In the case of 9-hydroxy-12-oxo-10*E*-dodecenoic acid, the first



SCHEME 2

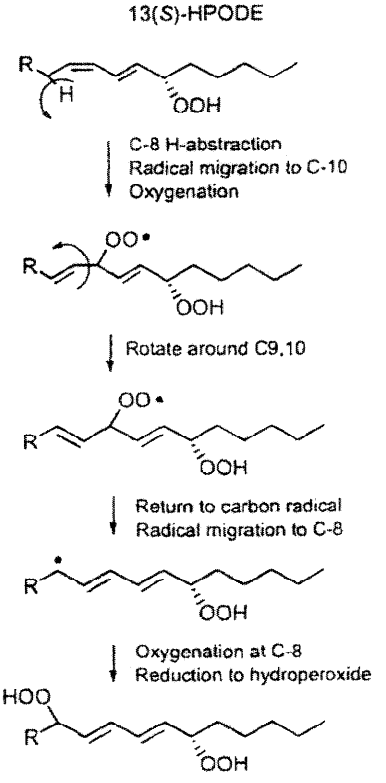


SCHEME 3

eluting enantiomer from the chiral column showed a negative split CD curve (extremum at 244 nm, $\Delta\epsilon$, −3.4) which defines *R* configuration for this enantiomer. Accordingly, the second enantiomer had a positive split CD curve (extrema at 244 nm, $\Delta\epsilon$, +3.2, and at 225 nm, $\Delta\epsilon$, −1.7), which reveals *S* configuration.

DISCUSSION

Analysis of the stereochemistry of 4-hydroxyalkenals formed from chiral hydroperoxides together with the detection of some



SCHEME 4

unusual dihydroperoxides of linoleic acid provide some valuable new insights into the pathways of 4-H(P)NE formation. We found that the 4-HPNE formed from 13*S*-HPODE largely retains the initial *S* configuration. This retention of configuration can be explained by the mechanism outlined in Scheme 1. The initial event is the abstraction of an allylic hydrogen at C-8 of 13*S*-HPODE. This yields a radical that can be localized on C-8, C-10, or C-12. Oxygenation at C-10 forms diastereomeric 10*R*,*S*,13*S*-dihydroperoxides. The formation of these products entails a bis-allylic oxygenation that is precedented in autoxidation (21) and in the soybean lipoxygenase-catalyzed oxygenation of a synthetic substrate, 16,17-dehydro-arachidonic acid (22). The doubly allylic 10-hydroperoxy group is unstable, and cleavage in a Hock rearrangement between C-9 and C-10 yields two aldehyde fragments, 9-oxo-nonanic acid and 4*S*-HPNE. The Hock rearrangement, which is promoted by protic and Lewis acids, occurs readily for hydroperoxides that have an unsaturated unit attached to the carbon bearing the hydroperoxide group (23, 24). Thus, benzylic, allylic, and dienylic hydroperoxides undergo the rearrangement readily by migration of the unsaturated group from carbon to oxygen while the weak O–O bond fragments. The initial 13*S*-hydroperoxy group of 13*S*-HPODE is not directly involved in this reaction sequence, and therefore the 4*S*-HPNE cleavage product retains its absolute configuration.

The 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid product (compound 1) from 13*S*-HPODE is formed through an alternative mechanism (Scheme 2). The first reaction is the Hock cleavage of 13-HPODE between carbons 12 and 13 (24). This yields the two aldehydes hexanal and 12-oxo-9*Z*-dodecenoic acid. Neither compound was detected in our analyses, because hexanal is volatile and virtually nondetectable in the UV, and 12-oxo-9*Z*-dodecenoic acid rapidly oxygenates to 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid (13, 25). The stereochemistry of the 9-hydroperoxy group is predicted to be racemic according to this mechanism (Scheme 2). Chiral analysis of the product

from 13*S*-HPODE (Fig. 8) revealed that it is a 53:47 mixture of the *S* to the *R* enantiomer, which is largely in agreement with this mechanism.

The reactions and products observed upon autoxidation of 9*S*-HPODE were mechanistically equivalent. In an initial Hock rearrangement, 9*S*-HPODE is cleaved into 9-oxo-nonanic acid and 3*Z*-nonenal. 3*Z*-Nonenal is very rapidly oxygenated to 4-HPNE, which in this case is formed as a racemic mixture (Fig. 7*B* and Scheme 3*A*). In plants, the cleavage of the 9*S*-hydroperoxide is catalyzed by a hydroperoxide lyase (26), and the subsequent nonenzymatic oxygenation of 3*Z*-nonenal has been described as the source for the formation of 4-HNE in plant tissue (13). There is also, however, an initial allylic hydrogen abstraction at C-14, which is analogous to the H abstraction at C-8 of 13-HPODE. The resulting radical rearranges and can be oxygenated at different positions (*i.e.* on carbons 10, 12, and 14). Hock cleavage of the 9*S*,12-dihydroperoxide yields 9*S*-hydroperoxy-12-oxo-10*E*-dodecenoic acid and hexanal (Scheme 3*B*). The predicted *S* configuration of the 9-hydroperoxy group was confirmed by chiral phase analysis (Fig. 8*B*).

An initial hydrogen abstraction at C-8 of 13*S*-HPODE, as postulated in Scheme 1, predicts the formation of positional isomers of diastereomeric linoleic acid dihydroperoxides. We identified a pair of diastereomeric 8,13-dihydroperoxides, providing evidence of the C-8 hydrogen abstraction. The corresponding 8,13-dihydroxy derivatives of linoleic acid have been characterized previously as minor end products of the heme-catalyzed degradation of 13*S*-HPODE (27). These dihydroxy derivatives were formed via synthesis of a leukotriene type of allylic epoxide (12,13-epoxy-8,10-octadecadienoic acid) that hydrolyzed to the 8,13-dihydroxides. This mechanism is quite distinct from the route to the 8,13-dihydroperoxides that were the major products under the conditions used in our study. The fact that these 8,13-dihydroperoxides have a *trans,trans* conjugated diene also provides strong circumstantial evidence for oxygenation at the C-10 position. Such a reaction at C-10 is required to account for the change in configuration of the original 9,10 *cis* double bond to the *trans* configuration found in the 8,13-dihydroperoxides (Scheme 4) (28, 29). The change in stereochemistry is allowed by the occurrence of a peroxyl radical at C-10. This permits rotation around the 9,10 bond. The subsequent loss of O_2 and formation again of the carbon radical aligns the carbon backbone in the more stable *trans,trans* configuration. Oxygenation at C-8 and formation of the hydroperoxide gives the stable 8,13-dihydroperoxide diastereomers that we isolated and identified (Scheme 4). The equivalent reactions occurred starting with 9*S*-HPODE, resulting in characterization of the two 9,14-dihydroperoxides by LC-MS and GC-MS.

The presence of α-tocopherol during the autoxidation of 13*S*-HPODE caused a slowing in the loss of this substrate and a noticeable change in the pattern of products. As an antioxidant, α-tocopherol intercepts peroxyl radicals and in the process forms an α-tocopheroxyl radical and the hydroperoxide. This accounts for the slowing of the disappearance of the 13*S*-HPODE starting material. The tocopheroxyl radical becomes the dominant free radical chain carrier, but it is not a sufficiently strong oxidant to abstract an allylic hydrogen at C-8 of 13-HPODE (30). This results in a selective absence of the dihydroperoxides discussed above and of the 4-HPNE cleavage product. On the other hand, direct Hock cleavage of the 13*S*-HPODE can still occur, providing 12-oxo-9*Z*-dodecenoic acid. The tocopheroxyl radical can abstract a doubly allylic hydrogen from C-11 of this intermediate, thus forming racemic 9-hydroperoxy-12-oxo-10*E*-dodecenoic acid as the major polar product of 13*S*-HPODE detected in the presence of α-tocopherol.

Interesting small deviations from the stereochemistries predicted in the mechanisms in Schemes 1 and 3 point to the existence of additional routes to the 4-hydroxyalkenals. We found that the 4-HPNE formed from 13*S*-HPODE was significantly less than 100% *S* in stereochemistry. The hydroperoxide starting materials were ≥98% *S* configuration, so ~10% of the *R* enantiomer was formed during the reaction by a pathway that is yet to be elucidated. Initial 9/13 hydroperoxide isomerizations occurring prior to chain cleavage may contribute to the product profiles. It was apparent also that the 4-HPNE formed from the 9*S*-HPODE was not racemic, but rather it showed a slight preference of the *S* enantiomer. In this case there must be some transfer of chirality from the 9*S* starting material to the 4-hydroperoxy product. The same consideration holds true for the formation of the 9-hydroperoxy-12-oxo-10*E*-dodecenoic acids; they showed similar small deviations from the predicted chiralities.

In conclusion, we provide evidence for at least two independent mechanisms leading from isomeric ω-6 fatty acid hydroperoxides to 4-H(P)NE. Here, we used the two isomeric linoleic acid hydroperoxides as model compounds. Analogous reactions are to be expected with hydroperoxides from other ω-6 fatty acids, especially arachidonic acid. With arachidonic acid, 11- and 15-hydroperoxy-eicosatetraenoic acid are the precursors to form 4-HPNE via the analogous mechanisms. The chiral analysis method of 4-HNE we developed here should be useful in further studies to investigate the mechanism of 4-hydroxyalkenal synthesis. For example, the possibility of an involvement of enzymes such as lipoxygenases or cytochrome P450s could result in biosynthesis of chiral 4-HNE and its analogues. A potential enzyme-initiated pathway to 4-HNE becomes increasingly important as physiological activities of 4-HNE in the sub-micromolar range are uncovered.

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Lipid Peroxidation Product-Mediated DNA Damage and Mutagenicity

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Abstract : Membrane lipid peroxidation processes yield products that may react with DNA to cause mutations. Lipid hydroperoxides from linoleic acid in the presence of transition metal ions caused strand breaks in plasmid DNA. DNA damage induced by reactive aldehydes known to be produced by decomposition of lipid hydroperoxides, such as 4-hydroxynonenal or malondialdehyde, was repaired by endonucleases and exonuclease III which resulted in the increase of single strand breaks in DNA. Lipid hydroperoxides as well as malondialdehyde and 4-hydroxynonenal also caused mutations in the pUC18 lacZ' gene when measured as a loss of α-complementation. In conclusion, the lipid peroxidation could be an important intermediary event in DNA damage and mutation by oxidative stress.

Key words : aldehydes, DNA damage, lipid peroxidation, mutagenicity

Oxidative modification of cellular constituents including lipids, proteins and DNA has been implicated in the etiology of different pathological conditions, such as diabetes, cataracts, pulmonary emphysema, arthritis, cancer, and in aging (Halliwell, 1987). In biological membranes lipid peroxidation is frequently a consequence of radical attack. The peroxidation of unsaturated fatty acids of cells produces many reactive species such as free radicals, hydroperoxides, and carbonyl compounds, which may cause damage to proteins and DNA (Cerutti, 1985). It has also been assumed that the decomposition of hydroperoxides mediated by catalytic transition metal ions may form much more toxic breakdown products such as alkoxy radicals (RO·), peroxyl radicals (ROO·), hydroxyl free radicals (·OH), and reactive aldehydes including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Slater, 1984; Cerutti, 1985; Ueda *et al.*, 1996). It is possible that in complex biological systems, oxygen free radicals and reactive aldehydes may cause protein and DNA damage indirectly by initiating lipid peroxidation, since polyunsaturated side chains of membrane lipids are especially susceptible to free radical-initiated oxidation (Fleming *et al.*, 1982).

Oxidative DNA damage is implicated in aging and age-related diseases such as cancer. While the ultimate mechanisms responsible for aging and aging-related diseases are obscure, there is growing evidence that oxygen radicals and lipid peroxidation are involved (Ames, 1983). In particular, lipid peroxidation is proposed to play a key role in membrane-mediated chromosomal damage (Cerutti, 1985). It has been shown that there is concurrent damage to not only lipids but also DNA during lipid peroxidation (Hruszkewycz, 1988; Fraga and Tappel, 1988). Several reports show that lipid hydroperoxides, including hydroperoxy-6,8,11,14-eicosatetraenoic acid and autoxidized methyl linolenate, caused DNA strand breaks, implicating the involvement of hydroxyl free radicals (Inouye, 1984; Ueda *et al.*, 1985; Weitberg and Corvese, 1989). A recent report demonstrates that autoxidized products of methyl linolenate and methyl arachidonate-enriched liposomes induce the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) (Park and Floyd, 1992), which is formed by hydroxyl radical or singlet oxygen attack at the C-8 position of the guanine base (Richter *et al.*, 1988; Park and Kim, 1994). It has also been shown that reactive aldehydes produced by the breakdown of lipid hydroperoxides mediate the formation of various DNA adducts such as pyrimido[1,2-α]purine-10(3*H*)-one (dGM_1) (Marnett *et al.*, 1986), N^6-oxopropenyl-2'-deoxyadenosine (dAM_1) (Chaudhary *et al.*, 1996), and $1,N^2$-ethenodeoxyguanosine (εdG) (Sodum and Chung, 1988) (Fig. 1). Therefore, a study of the relationship between lipid peroxidation and DNA damage is pertinent to understanding aging and carcinogenesis.

In this study, lipid peroxidation-mediated DNA dam-

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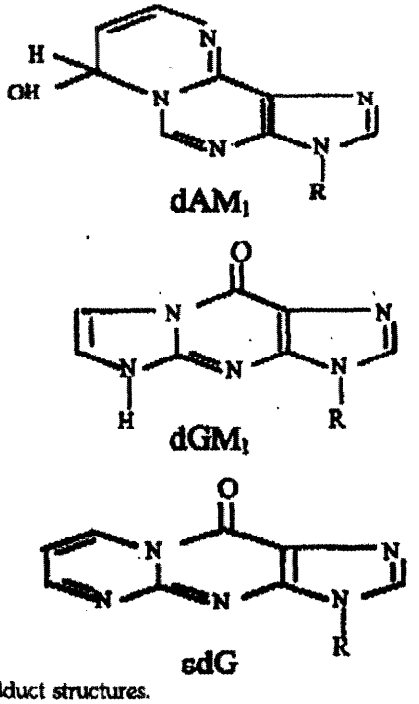

Fig. 1. Adduct structures.

age was investigated with purified lipid peroxidation products. DNA damage and mutation induced by lipid peroxidation products were examined by the induction of single strand breaks, susceptibilty of damaged DNA to repair enzymes and an *in vivo* mutagenicity assay. The results indicate that peroxidation of lipids may be an intermediary event in free radical-induced damage of DNA which presumably resulted in the mutation.

Materials and Methods

Materials

Chemicals and enzymes were obtained from the following sources: linoleic acid, diethylaminoethyl (DEAE)-cellulose, soybean lipoxygenase, 1,1,3,3-tetraethoxypropane (TEP), and isopropyl β-D-thiogalactopyranoside (IPTG) from Sigma Chemical Co. (St. Louis, USA); 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) from Promega (Madison, USA); 4-hydroxynonenal from Biomol (Plymouth Meeting, USA); and exonuclease III from Boehringer Mannheim (Mannheim, Germany).

Preparation of MDA

TEP (1 mmol) was dissolved in 90 ml of double-distilled water and hydrochloric acid (1 ml, 1 N) was added, and then the volume was brought to 100 ml with distilled water. A glass stopper was held firmly in place with parafilm to prevent loss of MDA while heating. After heating the flask in a water bath at 50°C for 60 min, it was cooled to room temperature and the appropriate stock solution was made. The exact concentration of the stock solution was confirmed by measuring its absorbance at 245 nm (ε=13,700 M^{-1} cm^{-1}) (Csallany *et al.*, 1984).

Preparation of lipid hydroperoxide

Freshly prepared ammonium salts of linoleic acid (13.5 μmol) were dispersed in 30 ml of 50 mM Tris-HCl buffer (pH 9.0) in a 250-ml Erlenmeyer flask equilibrated at 30°C. Oxygenation reactions were initiated by the addition of 0.65 ml of a freshly perpared solution containing 1 mg soybean lipoxygenase/ml in 50 mM Tris-HCl buffer, pH 9. After 2 min of incubation at 30°C a second addition of 0.65 ml of soybean lipoxygenase was made and the incubation continued at 30°C for an additional 8 min. Depletion of buffer oxygen in reactions containing excess lipoxygenase and unsaturated fatty acid was avoided by vigorous shaking of the incubation mixture. This procedure substituted for the need for oxygen-saturated buffer or continuous aeration of the reaction mixture during catalysis. Reactions were terminated by the addition of 7.5 ml of ethanol and cooled to 4°C on ice. Reaction mixtures were acidified (pH 3.0) and applied in 5-ml portions to 6-ml C_{18} reversed-phase sample preparation columns (J.T. Baker) which had been previously prewashed sequentially with 25 ml of HPLC-grade ethanol and 25 ml water. The columns were washed sequentially with 25 ml of 20% ethanol in water and 50 ml water to remove proteinaceous material and 10 ml hexane to remove trapped water. Fatty acid hydroperoxides were eluted with 10 ml of methyl formate. Methyl formate fractions were collected and stored at -70°C until used for further experiments (Graff *et al.*, 1990).

Preparation of crude endonucleases

Endonuclease-containing crude extracts from *E.coli* K-12, from which DNA had been removed by DEAE-cellulose chromatography, were prepared according to Riazzudin (1980). Before use, the extracts were diluted with BE buffer (20 mM Tris-HCl, pH 7.5/100 mM NaCl/15 mM EDTA) to a final protein concentration of 1.2 mg/ml.

DNA isolation

Plasmid DNA was prepared from bacteria harboring the plasmid pBluescript or pUC18 (Sambrook *et al.*, 1989). Bacteria were incubated overnight at 37°C with good aeration and shaking. Cells were decanted into a 1.5 ml microcentrifuge tube and collected by centrifugation at 12,000×g for 30 s at 4°C. After removing the supernatant, the pellet was suspended in 100 μl of ice-cold solution I (50 mM glucose/25 mM Tris-Cl, pH 8.0/10

mM EDTA) and 200 μl of freshly prepared solution II (0.2 N NaOH/1% SDS) was added. The contents were mixed by inverting the tube rapidly five times. To this mixture 150 μl of ice-cold solution III (60 ml 5 M potassium acetate/11.5 ml glacial acetic acid/28.5 ml H_2O) were added. After incubation on ice for 3-5 min, the mixture was centrifuged at 12,000×g for 5 min and then the supernatant was transferred to a fresh tube. After the extraction of this solution with an equal volume of phenol/chloroform, DNA was recovered by the addition of 2 volumes of ethanol (-20°C).

Agarose gel electrophoresis

In a typical experiment, 1 μg pBluescript plasmid DNA was incubated with MDA (2.5 mM), HNE (10 mM), or linoleate hydroperoxide (50 μM)/$FeCl_3$ (50 μM) in 5 mM phosphate buffer, pH 7.4. In most cases incubations were at 37°C for 2 h. The reactions were terminated by an addition of cold ethanol and DNA was collected by centrifugation. The treated DNA was incubated with 5 μl of crude endonuclease (1.2 mg/ml) in endonuclease buffer solution (40 mM Tris-HCl, pH 8.0/200 mM NaCl/2 mM EDTA), or exonuclease III (2000 units) in exonuclease buffer solution (40 mM Tris-HCl, pH 8.0/200 mM NaCl/30 mM $CaCl_2$) at 37°C for 2 h. After incubation, 1.5 μl of 10% SDS and 3.3 μl of 6X dye solution were added to each reaction mixture and the samples were subjected to agarose gel electrophoresis. Following electrophoresis, gels were stained with ethidium bromide, irradiated from below with a UV transilluminator box, and photographed. The relative fluorescence intensities of the open circular forms of plasmid were quantitated by measuring the area of a densitometer tracing (Park and Floyd, 1994).

Mutagenicity assay

Exponentially growing *E.coli* JM109 host cells (OD_{600} =0.4) were chilled and pelleted by centrifugation at 12,000×g for 10 min at 4°C. Transformation protocols were performed as described (Sambrook *et al.*, 1989) which are summarized in Fig. 2. The treated pUC18 DNA with same conditions as samples for agarose gel electrophoresis was incubated with 0.2 ml of competent cells at 0°C for 30 min and then the mixture was heat-shocked at 42°C. After 2 min, LB medium was added and incubated at 37°C for 1 h. Transfected cells were plated on LB agar plates containing 0.8 mg of X-gal and 0.8 mg of IPTG and 0.01% ampicillin. The plates were inverted and incubated for 24 h at 37°C before counting colonies.

Replicates

Each result described in this paper is representative of three separate experiments.

Results

We investigated lipid peroxidation product-mediated DNA damage by an increase in the susceptibility of damaged DNA for repair enzymes such as endonuclease and exonuclease III, which acts as an AP endonuclease. Plasmid DNA (pBluescript or pUC18) was treated with MDA or HNE at 37°C for 2 h; the damaged DNA was recovered by ethanol precipitation and redissolved in water. The treated plasmid DNA was incubated with crude endonuclease (1.2 mg/ml) or exonuclease III (2000 units). An increase of strand breaks in DNA which resulted in the repair activity of endonuclease and exonuclease III was measured by agarose gel electrophoresis. While exonuclease III is known to recognize sites of base loss (AP-sites) specifically (Rogers and Weiss, 1980), the repair enzyme-containing crude extracts from *E. coli* contain glycosylases and endonucleases recognizing a variety of base modifications (Epe

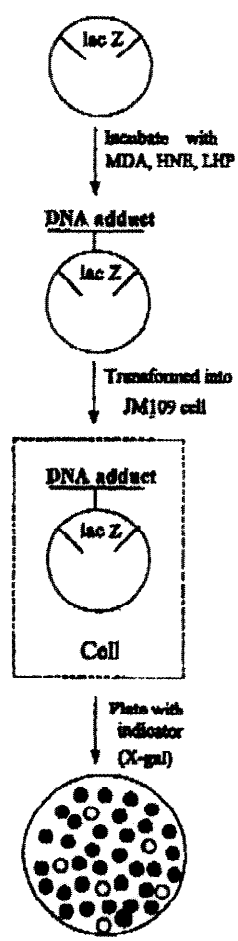

Fig. 2. *In vivo* assay for the mutagenicity of lipid peroxidation products.

et al., 1988). It has been shown that repair enzymes can be used as sensitive probes to analyze DNA damage. The assay uses supercoiled DNA as a target, in which both strand breaks and the incision of repair enzymes (endonuclease-sensitive sites) can be easily detected and quantitated (Epe *et al.*, 1989). When MDA-modified DNA was incubated with repair enzymes, single strand breaks reflected by the conversion of covalently closed circular double-stranded supercoiled DNA (ccc) to open (relaxed) circular double-stranded DNA (oc) were significantly increased (Fig. 3). In contrast, untreated DNA induced no significant increase in strand breaks with treatment of repair enzymes. However, there was only a small increase of single strand breaks in the HNE-treated DNA after incubation with endonuclease, as shown in Fig. 4. This result may indicate that MDA causes more extensive damage to DNA compared to HNE. As shown in Fig. 5, the extensive strand breaks of DNA were observed with linoleate hydroperoxide/Fe^{3+} even without treatment of repair enzymes, which may be caused by the direct attack of hydroxyl radicals generated from the breakdown of lipid hydroperoxide.

To examine the mutagenicity of lipid peroxidation products, pUC18 plasmid DNA was treated with MDA, HNE, or linoleate hydroperoxide/Fe^{3+}, and then damaged DNA was recovered. *E. coli* (JM109 competent cells) spheroplasts were transformed with treated DNA. *E. coli* expressing fully active β-galactosidase produce dark blue colonies on the indicator substrate X-gal, whereas mutation within the lacZ' α segment of pUC18 DNA resulted in the expression of much less active β-

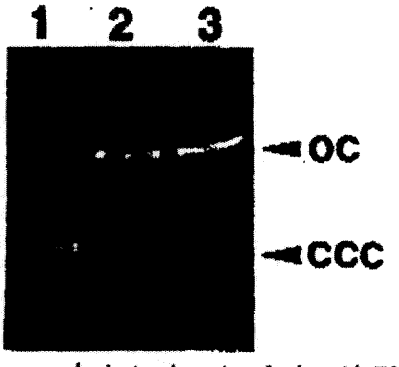

Fig. 3. Agarose gel electrophoresis of plasmid DNA treated with MDA. Reactions were carried out for 2 h at 37°C and then damaged DNA was further incubated with repair enzymes for 2 h at 37°C. Lanes correspond to reaction conditions as follows: 1, no repair enzymes; 2, + endonucleases; 3, + exonuclease III. ccc, covalently closed circular DNA; oc, open circular DNA.

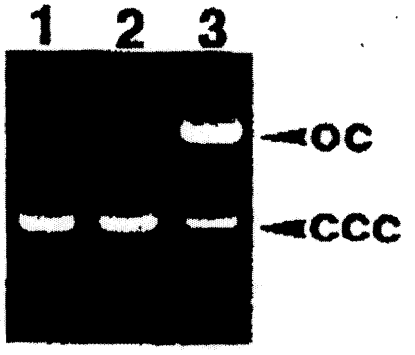

Fig. 5. Cleavage of supercoiled pBluescript plasmid DNA in the presence of linoleate hydroperoxide/Fe^{3+}. Lanes correspond to reaction conditions as follows: 1, DNA; 2, DNA + Fe^{3+}; 3, DNA+linoleate hydroperoxide/Fe^{3+}

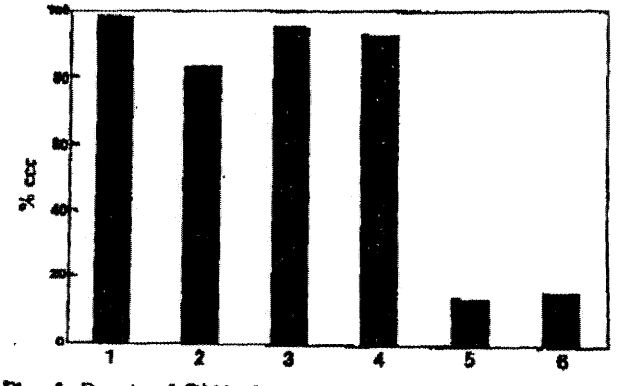

Fig. 4. Repair of DNA damage induced by HNE and MDA. The damaged DNA was treated with repair enzymes and was analyzed by agarose gel electrophoresis and the amount of uncleaved ccc DNA was measured. 1, HNE-treated DNA; 2, HNE-treated DNA + endonucleases; 3, HNE-treated DNA + exonuclease III; 4, MDA-treated DNA; 5, MDA-treated DNA + endonucleases; 6, MDA-treated DNA + exonuclease III. The amount of ccc DNA of untreated DNA was used as 100%.

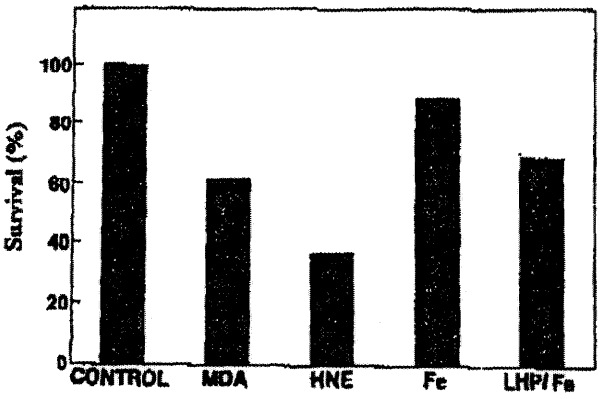

Fig. 6. Transforming ability of lipid peroxidation product-treated plasmid DNA in *E. coli* JM109. Damaged DNA as described in the Material and Methods was transfected into competent bacteria. Transformants were selected on media containing ampicillin and the colonies were scored after overnight incubation at 37°C. The relative survival was determined by the ratio between the number of colonies obtained with treated and untreated DNA.

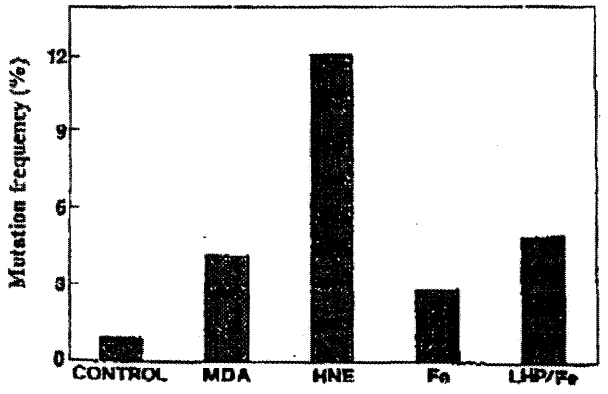

Fig. 7. Mutagenicity of lipid peroxidation products-treated plasmid DNA in *E. coli* JM109. Transfection was carried out as described in Fig. 6. Mutagenic frequency was expressed as the ratio between the mutant colonies and total colonies.

galactosidase and yields light blue or white colonies. The loss in viability (survival of colony-forming ability) of pUC18 plasmid DNA upon incubation with lipid peroxidation products was in a range of 40-60% compared to untreated DNA (Fig. 6). The frequency of mutation induced by lipid peroxidation products was 4.5-12%, as shown in Fig. 7. The results indicate that both linoleate hydroperoxide/Fe^{3+} and reactive aldehydes form mutagenic lesions in DNA. Although MDA-treated DNA formed a significantly higher level of lesions susceptible to repair enzymes, a much lower mutation frequency was observed compared to HNE-treated DNA. This implies that HNE creates highly mutagenic lesions in DNA. Fe^{3+} itself induced mutagenic lesions in DNA, which is consistent with previous findings with iron-treated ϕX174 *am3* (amber 3 mutation) DNA (Loeb *et al.*, 1988). Addition of linoleate hydroperoxide moderately increased mutagenic frequency (Fig. 7).

Discussion

It has been proposed that lipid peroxidation is a continual process in living aerobic cells, is maintained at a low level, and can be prevented from entering into the autocatalytic phase by protective enzymes and antioxidants (Munkres, 1976). Chemical and physical agents that enhance membrane free radical reactions may accelerate this process beyond the capabilities of the protective systems and thus cause widespread lipid peroxidation (Lee and Park, 1995).

The DNA-damaging action of peroxidized lipids may contribute to disorders in genetic information transfer; thus, peroxidized lipids act as etiological agents in biological aging and tumorigenesis (Ueda *et al.*, 1985). Consequently, the study of DNA damage under oxidative stress and its relationship to lipid peroxidation is relevant. While observation of lipid peroxidation-induced damage to DNA is limited and indirect, it has been proposed that lipid peroxidation is a major mode of membrane-mediated chromosomal damage (Cerutti, 1985). A major problem in elucidating the mechanisms behind the proposed genotoxicity of the lipid peroxidation process is the discrimination between DNA damage caused by the direct action of free radicals and their role in the initiation of lipid peroxidation and the damage caused by the reaction of DNA with the final products of the lipid peroxidation process (Vaca *et al.*, 1988). Several reports demonstrate that hydroperoxides can cause DNA strand breaks (Inouye, 1984; Ueda *et al.*, 1985 Hruszkewycz, 1988). One report shows that lipid peroxide interaction with guanine nucleotide causes double-strand DNA breaks specifically (Ueda *et al.*, 1985), but the mechanism is not known and the DNA adducts have not been identified. Others have demonstrated that peroxidizing arachidonic acid causes structural changes in isolated DNA (Reiss and Tappel, 1973).

Aldehyde compounds such as MDA and HNE, together with similar compounds generated in lower amount such as 4-hydroxynonenal, are associated with lipid peroxidation (Esterbauer *et al.*, 1991). MDA undergoes reactions with DNA and a decrease in template activity of liver DNA from rats fed with MDA was observed (Klamerth and Levinsky, 1969). It has been also shown that MDA is mutagenic in certain strains of *Salmonella typhimurium* (Basu and Marnett, 1983) and murine L 5178 Y lymphoma cells (Yau, 1979). HNE has a conjugated aldehyde structure, which enables it to react readily with DNA, and is 4000 times more efficient than MDA in inducing an SOS response in *S. typhimurium* (Benamira and Marnett, 1992). Consequently HNE, together with MDA and other aldehydes, have been proposed to be partly responsible for the mutagenic properties of lipid peroxidation (Esterbauer *et al.*, 1990).

The reaction of secondary free radicals and reactive aldehydes, which are produced from the breakdown of unstable lipid hydroperoxide, with DNA is likely to be important in the overall action of these toxic species. We exposed purified plasmid DNA to lipid peroxidation products *in vitro* and measured alterations of biological activity. Present results clearly indicate that lipid peroxidation products are highly mutagenic. Therefore, lipid peroxidation may represent an important intermediary step in the process of oxygen radical-induced genetic damage.

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Critical Review

4-Hydroxynonenal in the Pathomechanisms of Oxidative Stress

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Summary

Here we review the current knowledge on the biochemistry and molecular pathology of oxidative stress with specific regard to a major aldehydic end-product stemming from peroxidation of biomembranes, that is 4-hydroxynonenal (HNE). This multifunctional molecule, which derives from the most represented class of polyunsaturated fatty acids in the membranes, is potentially able to undergo a number of reactions with proteins, phospholipids, and nucleic acids. Despite an active metabolism in most of the cell types, HNE can be detected in several biological tissues by means of sufficiently precise methods, although with different sensitivity. In particular, relatively high steady-state levels of HNE are often detectable in a large variety of human disease processes, pointing to some involvement of the aldehyde in their pathogenesis. Among the prominent pathobiochemical effects of HNE is its remarkable stimulation of fibrogenesis and inflammation, which indicates a potential contribution of the aldehyde to the pathogenesis of several chronic diseases, whose progression is indeed supported by inflammatory reactions and characterized by fibrosis. Further, of interest appears to be the ability of HNE to modulate cell proliferation through interference with the activity of cyclins and protein kinases and with the apoptotic machinery. Finally, on the basis of the already achieved evidence, pursuing investigation of the role of HNE in signal transduction and gene expression seems very promising.

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Keywords Chemical reactivity; fibrogenesis; 4-hydroxynonenal; inflammation; metabolism; signal transduction; oxidative stress.

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Abbreviations: HNE, 4-hydroxynonenal; ROS, reactive oxygen species; DHN, 1,4-dihydroxynon-2-ene; GSH, glutathione; GST, glutathione-S-transferase; HNA, 4-hydroxy-nonenoic acid; PUFA, polyunsaturated fatty acid; PE phosphatidylethanolamine; TGFβ1, transforming growth factor β1; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TNFα, tumor necrosis factor α; JNK, cJun amino-terminal kinase; ERK, extracellular signal regulated kinase; MAPK, mitogen activated protein kinase; PKC, protein kinase C; MCP1, monocyte chemotactic protein-1; AP-1, activator protein 1; NF-κB nuclear factor kappa B.

This paper is dedicated to Professor Hermann Esterbauer (Graz, Austria).

INTRODUCTION

This review primarily deals with the pathobiochemical effects of the lipid-derived aldehyde, 4-hydroxy-trans-2-nonenal (HNE), which at the time of its discovery in natural fats by Hermann Esterbauer (*1*) was regarded as a mere byproduct of autoxidation of unsaturated fatty acids, when triglycerides become rancid. The situation changed when it turned out that HNE is a normal constituent of mammalian tissue membranes (*2*) and recent interest is not only based on the fact that HNE is a specific marker of oxidative stress (*3*)—i.e., the prevalence within the cell of oxidizing species over the cellular antioxidant potential—but evidence is growing also that HNE is a (patho)physiological modulator of several signal transduction processes (*4*) and might be causally involved in the pathogenesis of a great number of inflammatory and degenerative diseases which steadily show increased levels of free radical–mediated reactions.

Because the biochemistry of HNE has been extensively reviewed in 1991 by Esterbauer et al. (*5*), here we focus on recent evidence pointing to a pathophysiological role of the aldehyde during oxidative stress.

HNE, A PRODUCT OF MEMBRANE OXIDATIVE BREAKDOWN

Formation

The aldehyde derives from n-6 polyunsaturated fatty acids (PUFAs), like linoleic acid, linolenic acid, and arachidonic acid,

containing a C=C double bond in position 6 when counted from the methyl end. The most efficient precursor of this PUFA family for the formation of HNE is arachidonic acid (*6*). It is noteworthy that despite several theoretical considerations about the chemical mechanism of HNE formation (*7*) and also some experimental efforts (*8*), the precise route of the transformation of PUFAs to HNE has not yet been elucidated. It is generally assumed that it is a non-enzymatic free radical process yielding hydroperoxides as primary products which then decompose to HNE by fragmentation, but Esterbauer et al. (*8*) have shown that HNE formation from arachidonic acid is greatly enhanced in the presence of a NADPH-linked microsomal system. The decomposition of hydroperoxides is promoted by iron ions. Recently, it has been proposed that the formation of HNE from PUFAs might be catalyzed by cytochrome P450. HNE was found to be markedly increased in Hep G2 cells overexpressing human cytochrome P450 2E1 (*9*).

Metabolism

Three alternative routes were originally proposed for the metabolism of HNE: reduction of the carbonyl group to an alcohol, oxidation to the corresponding carbonic acid and conjugation to glutathione (GSH) via Michael addition of the thiol group of GSH. Research in the last decade has shown that the metabolism is more complex, involving also the proteasome pathway of HNE-protein adducts (*10*). In mice, a product of ω-hydroxylation of HNE, 4,9-dihydroxy-2-nonenoic acid, has been identified (*11*), which is probably formed via a cytochrome P450-catalyzed oxidation of the primary product 4-hydroxynonenoic acid.

Of high significance might be the finding that HNE can influence its metabolism both at the enzymatic and the transcriptional level. For example, for the metabolic enzyme glutathione-S-transferase (GST), it has been shown that it is inhibited as well as induced by HNE. The human GST isoform P1-1 is reversibly inhibited by HNE (*12*). Cysteine 47 is one of the major targets in this enzyme, forming a Michael adduct with HNE. On the other hand, HNE is an inducer of the same isoform P of GST, at least in rat liver epithelial cells (*13*). It was suggested that the induction of GST-P by HNE may represent an important cellular defense mechanism against oxidative injury.

Measurement

A huge number of methods has been developed for the determination of either free HNE or its conjugation products with biomolecules (*14*). Most frequently polyclonal or monoclonal antibodies are now applied, which are directed against HNE-protein conjugates, but no procedure exists that would fulfill all the requirements of an ideal analytical method. Thus, it depends mostly on the purpose and the available facilities, whether a photometric assay (*15*) is used, which is simple and suitable for routine analysis but determines hydroxyalkenals in general, rather than HNE, or a highly sophisticated mass spectrometric method [electrospray MS with HNE-d(*11*) as internal standard] (*16*), or stereoanalysis by gas chromatography (*17*) is chosen.

Taking advantage of these analytical methods, steady-state level of HNE was determined in many tissues and body fluids. In general terms, the biological occurrence of the hydroxyalkenal appears within the range of 0.1–1 μM (*5*). Under defined pathological processes, HNE steady-state concentration within membranes can easily reach 5–10 μM (*18*) or more (*19*), despite an active cellular metabolism, because of its high lipid/water compartmentalization coefficient (*5*).

HNE REACTIONS WITH BIOMOLECULES

Structure–Reactivity Relationship

An overview of the diverse reactions of HNE is shown in Figure 1. HNE is an extraordinary compound containing three functional groups that in many cases act in concert and help to explain its high reactivity. Most importantly, there is a conjugated system consisting of a C=C double bond and a C=O carbonyl group in HNE. The hydroxy group at carbon 4 contributes to the reactivity both by polarizing the C=C bond and by facilitating internal cyclisation reactions such as thio-acetal formation.

Virtually all of the biochemical effects of HNE can be explained by its high reactivity towards thiol and amino groups. Among the primary reactants for HNE are the amino acids cysteine, histidine, and lysine, which—either free or protein-bound—undergo readily Michael addition reactions to the C=C double bond (Fig. 1). After this primary reaction, which confers rotational freedom to the C_2—C_3 bond, secondary reactions may occur involving the carbonyl and the hydroxy group. Primary amino groups may alternatively react with the carbonyl group to form Schiff bases.

Biophysical Effects

Besides biochemical effects, biophysical changes of protein and lipid membrane conformation have to be considered. It appears that these effects are differentially dose-dependent. While significant protein conformational changes have been observed with synaptosomal membranes already at a physiologically relevant concentration of 1 μM HNE, membrane fluidity increased only at a much higher concentration (50 μM) (*20*). If membrane disruption occurs, this may be the cause of modified membrane protein function like decreased Na^+/K^+ ion pump activity (*21*).

Reaction with Proteins

The conjugation with protein-bound amino acids does not randomly occur (*12*). If the amino acid residues are in the active site of an enzyme, the activity of this protein is frequently diminished. This inhibition is in some case reversible in presence of thiol-containing compounds such as glutathione (GSH) (*12, 22*)

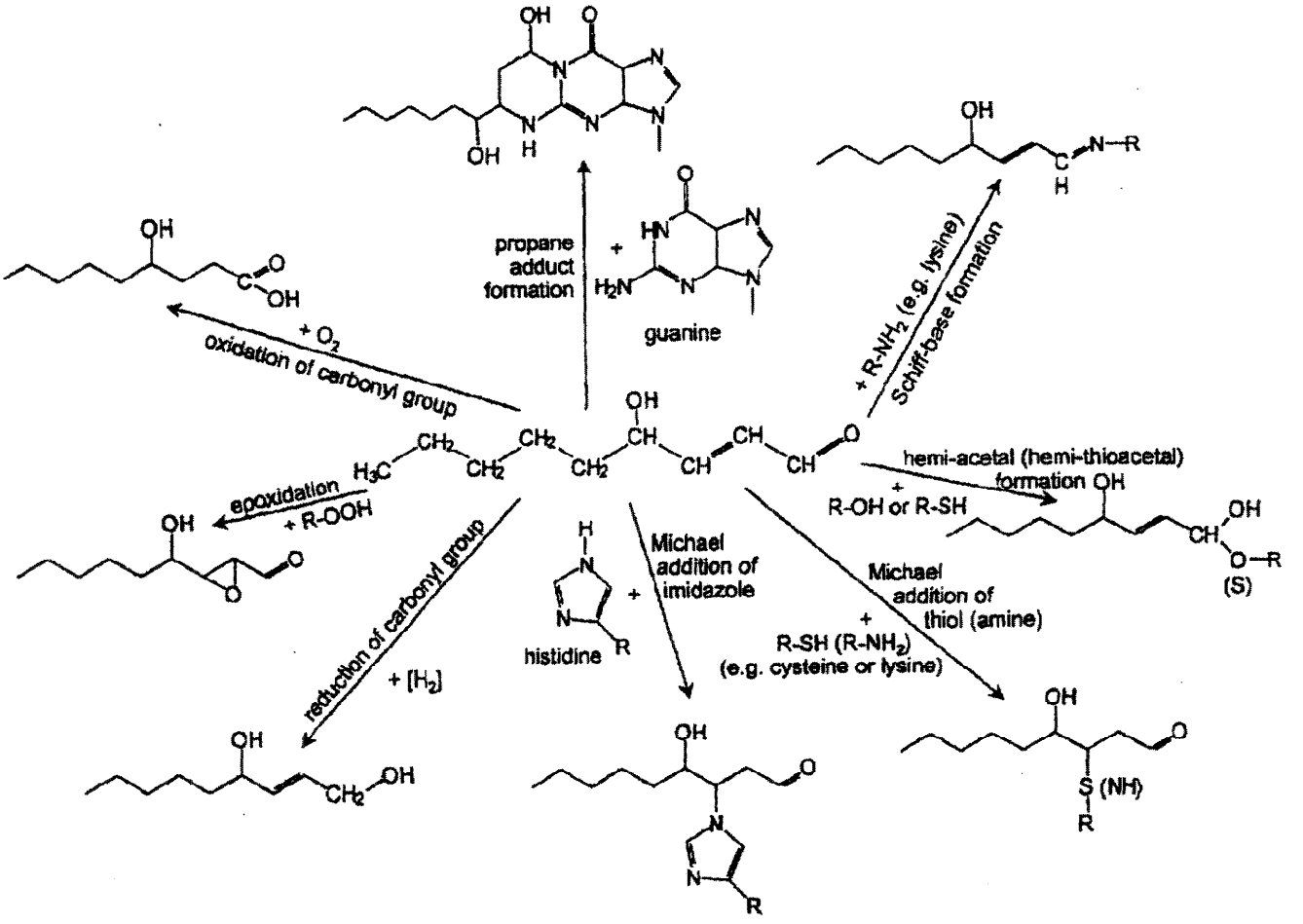

Figure 1. Overview of the reactions of 4-hydroxynonenal with different biomolecules.

or acetyl cysteine, but in most instances irreversible destruction takes place (*23*).

Interestingly, at submicromolar concentrations of the aldehyde, activation rather than inactivation of enzymes has been reported. Rat hepatocyte protein kinase C (PKC) βI and, to a much greater extent, PKC βII activities were markedly increased by 0.1 μM HNE, whereas they were unaffected or even inhibited by 1 to 10 μM HNE (*24*). Very recently, selective activation of PKC isoforms βI, βII by 0.1–1 μM HNE was also found in NT2 neuronal cells, in parallel to a marked increase of intracellular amyloid β production (*25*).

The reaction of HNE with proteins is frequently associated with their cross-linking, leading to the formation of fluorophores. A major fluorophore has been identified as a lysine-derived dihydropyrrol derivative: a 2-alkyl-2-hydroxy-1,2-dihydropyrrol-3-one iminium cross-link is formed in the reaction of HNE with lysine in a 1:2 ratio (*26*).

Moreover, because HNE tends to concentrate in biomembranes rather than into the aqueous space of cells, it can be assumed that enzymes embedded in membranes are more prone to attack by HNE than cytosolic enzymes. A consequential consideration is that the concentration of exogenous HNE needed to produce a significant biochemical effect can be orders of magnitude higher compared to the endogenous steady state level of HNE (*19*).

Reactions with Phospholipids

Not only proteins and peptides such as the histidine-containing dipeptide carnosine (*27*), but also amino group-containing phospholipids and nucleic acids are targets of HNE. Among the phospholipids containing an amino moiety, phosphatidylethanolamine (PE) was found to be a good target for HNE, while phosphatidylserine reacted poorly. The main resulting compounds with PE were a Michael adduct plus a minor Schiff base adduct, which was partly cycled as a pyrrole derivative (*28*).

Reactions with Nucleic Acids

The genotoxic potential of HNE has been studied, e.g., for hepatocytes and cerebral endothelial cells (ECs) (*29*). HNE at a concentration of 1 μM caused elevated levels of chromosomal aberrations and at 10 μM the formation of micronuclei in

CCs. HNE is thought to contribute to the low level of DNA adducts which are abundant both in untreated rodent and human genomes (*30*). Two pathways are considered to be responsible for the mutagenicity of HNE. One is the formation of 1, *N*-2-propano-deoxyguanosine derivatives by direct interaction with the guanosine moiety of DNA. Reaction of HNE with calf thymus DNA resulted in a pair of diastereomeric adducts, with one adduct predominately formed with a modification level of 1.2 +/− 0.5 adducts/10^7 nucleotides (*31*). A second route putatively involves the oxidation of HNE to the epoxy derivative, which has been shown to yield etheno adducts in vitro (*32*). These etheno adducts have also been found in human lung tissue (*33*).

EFFECTS ON SIGNAL TRANSDUCTION AND GENE EXPRESSION

Over the last few years, a defined area of free radical research has developed very much, namely that focused on the modulation of redox-sensitive transcription factors by ROS and related reactive chemical species.

As regards the transcription factor activator protein 1 (AP-1), which regulates the expression of a great variety of genes involved in cell proliferation, differentiation, and function (*34*), HNE has consistently shown a strong up-regulating effect. In fact, concentrations of the aldehyde within the pathophysiological range (1–10 μM) markedly enhance AP-1 nuclear binding in different cytotypes, including murine and human macrophages, human hepatic stellate cells, rat hepatocytes, and cortical neurons (*35* and references herein).

To exert such an effect, HNE signaling to the nucleus may involve, at the plasma membrane level, the activation of the EGF receptor (*36, 37*) and/or PDGF receptor (*38*). Such evidence is providing a mechanism for both paracrine and autocrine regulatory loops that are potentially induced by this highly diffusable aldehyde, even if direct membrane diffusion in a lipophilic vehicle is also probable. Independently from the triggering mechanism, HNE in the low micromolar range (10^{-5}–10^{-6} M) is able to markedly activate novel isoforms of protein kinase C (nPKCs), in particular the δ isoform (*24*). Further, cell co-treatment with specific inhibitors of novel PKCs, like rottlerin, allows to largely prevent HNE-induced AP-1 nuclear binding (Poli et al, submitted for publication). Along the pathway of AP-1 activation, as already demonstrated on different cytotypes, the aldehyde involves c-Jun amino-terminal kinases (JNKs) rather than extracellular signal regulated kinases (ERKs) (*39–41*).

While experiments are in progress to fully characterize HNE signaling through mitogen-activated protein kinase (MAPK) cascade, confocal microscopy analysis let us to prove that 4-hydroxynonenal, externally added to macrophages, hepatocytes, or stellate cells, tends to concentrate in discrete areas of the nucleus (*35, 39*). Such evidence if on one hand does not exclude the possibility of HNE interaction with JNKs with consequent nuclear translocation, as already demonstrated for stellate cells (*39*), on the other hand, it points to a totally unexplored aspect of HNE biology that is in situ reactivity with nuclear targets.

In relation to another well-studied redox-sensitive transcription factor, nuclear factor kappa B (NF-κB), HNE showed different effects depending on the cell type or even cell line involved. That is the case of cells of the macrophage lineage in which the aldehyde either inhibited (*42*) or did not change the nuclear translocation of the peptide (*43*). On the contrary, in neuronal cells HNE consistently downregulates NF-κB nuclear binding (Camandola et al., submitted for publication).

Stimulation of AP-1 nuclear binding by HNE actually leads to overexpression of a number of genes that have AP-1 consensus sequences in their promoter regions. At doses compatible to those detectable in vivo the hydroxyalkenal induces expression and synthesis of collagen type I (*44*), transforming growth factor β1 (TGFβ1) (*45*), aldose reductase (*46*), and gamma-glutamylcysteine synthetase (*47*).

PATHOBIOCHEMICAL EFFECTS OF HNE

Excessive Fibrogenesis

A pathological event clearly related to HNE and lipid peroxidation is that of the accumulation of connective tissue, a process termed fibrosis. Practically all causes of fibrosis, in various organs (liver, lung, kidney, arterial wall, etc.) recognize the potential involvement of reactive oxygen species (ROS) and related products, in particular aldehydes stemming from membrane oxidative breakdown (*48, 49*).

From the analysis of the various pathomechanisms underlying the process of fibrotic degeneration of a given tissue, the role of ROS and aldehydes like HNE as stimulators of signal transduction and second messengers appears prominent throughout the whole disease process. Outstanding is the HNE-induced modulation of expression and synthesis of the main fibrogenic cytokine, the transforming growth factor β1 (TGFβ1), by macrophages (*45*). This cytokine is indeed involved in triggering fibrosis since it allows activated phagocytes to cross-talk with extracellular matrix producing cells. Further, at least in the fibrotic liver, HNE has also been demonstrated to markedly stimulate collagen production by hepatic stellate cells (*44*).

Because of the recent wide availability of monoclonal antibodies raised against HNE-histidine adducts, there is an increasing number of reports about the detection of this aldehyde in a variety of human diseases. In particular, increased amounts of HNE have been proved in different chronic liver diseases, including alcoholic hepatitis, hemochromatosis, and primary biliary disease (*50*), all characterized by progressive fibrosis. In addition, HNE accumulation has been shown in the most common fibrotic lesion, i.e., the atherosclerotic plaque (*51*), and, more recently, in fibrotic proliferative retinopathies (*52*).

Inflammatory Reactions

Inflammation actually represents the main mechanism of perpetuation of fibrosis in chronic diseases. On one hand, activation of phagocytes provides fibrogenic stimuli, on the other hand,

the same event leads to overexpression of a variety of cytokines, growth factors and chemokines, provided with pro-inflammatory activity.

Besides up-regulation of the inflammatory and fibrogenic cytokine TGFβ1, HNE has recently been shown to induce expression and synthesis of monocyte chemotactic protein-1 (MCP1) (*53*). Such finding provides new emphasis to the relatively old evidence of a direct chemotactic effect on neutrophils exerted by the aldehyde, first described in vitro by Curzio et al. (*54*, see also ref. *4*), and later on confirmed in vivo by Schaur et al. (*55*).

On the contrary, at least regarding production of tumor necrosis factor α (TNFα) by monocytic cells in culture, HNE was found strongly inhibitory, since it is able to interfere with the nuclear translocation of the transcription factor-κB (NF-κB) (*42*), essential for optimal transcription of a number of inflammatory cytokines.

Together with other PUFA oxidation products, HNE has been detected in patients with adult respiratory distress syndrome (*56*), or individuals exposed to ozone (*57*). The aldehyde appears to contribute to heat shock protein expression by ozone-treated macrophages (*58*) and to the apoptotic insult made by this air pollutant (*57*).

Present findings already suggest a potential involvement of HNE in chemotactic recruitment of phagocytic cells and their activation, both in acute and chronic inflammatory processes, but its actual contribution to the modulation of steady-state levels of chemical mediators is yet largely unexplained.

Inhibition of Cell Proliferation, Apoptosis

HNE has shown a pronounced effect on the proliferation of cancer cells (see ref. *4*). For example, the aldehyde, at concentrations similar to those found in normal cells, blocked proliferation and induced a granulocyte-like differentiation in the leukemic HL-60 cell line (*59*). These effects were accompanied by a marked increase in the percent of cells in the G0/G1 phase of the cell cycle. Among the possible mechanisms of action, a modulation of cell cycle regulator proteins by HNE was then investigated. Indeed, HNE was able to downregulate the expression of the cyclins D1, D2, and A, while the cyclins B and E, and the cyclin-dependent kinases CDK2 and CDK4 remained unaffected (*59*).

Another way for HNE to contribute to cell cycle control may be through its pro-apoptotic effect, already demonstrated to be exerted in different cytotypes such as neurons (*60*), endothelial cells (*6*), hepatocytes (Poli et al., unpublished data). HNE-induced apoptosis occurs with aldehyde concentrations not higher than 0.1–10 μM and, depending on the cell type, it may involve activation of JNK and AP-1 and simultaneous inactivation of NF-κB.

Neuronal Degeneration

An important focus of current HNE research is directed towards its role in neuronal degeneration that occurs in various neurodegenerative disorders, in particular Alzheimer's disease (*61*) and Parkinson's disease (*62*).

Table 1
List of human disease processes in which increased steady-state levels of the aldehyde 4-hydroxynonenal have been detected

Adult respiratory distress syndrome	Genetic hemochromatosis
Alzheimer's disease	Ischemic hepatitis
Amyotrophic lateral sclerosis	Liver transplantation
Atherosclerosis	Lupus erythematosus
Chronic alcoholic disease	Mitochondrial complex 1 deficiency
Chronic hepatitis C	Parkinson's disease
Chronic iron overload	Premature chronic lung disease
Chronic exposure to ozone	Primary biliary cirrhosis
Crohn's disease	Proliferative vitreoretinopathies
Circulatory shock	Rheumatoid arthritis
Diabetes mellitus	Systemic amyloidosis
Deep venous thrombosis	Wilson's disease

At the plasma membrane of neuronal cells, HNE can modify release and uptake of neurotransmitters, ion channel activity, function of ion-motive ATPases and glucose transporters, and coupling of cell-surface receptors to GTP-binding proteins. HNE can also impair mitochondrial functions and promote a cascade of events that culminates in apoptotic cell death (*61–63*).

Further, with special regard to Alzheimer's disease, in Neuro 2A cells, HNE was shown to exert its damaging effects also at the level of the microtubular system, through Michael addition products involving tubulin, with eventual microtubule derangement and alteration of neurite outgrowth (*64*).

CONCLUDING REMARKS

Reactive molecules stemming from free radical-mediated oxidative breakdown of biomembrane lipids most likely contribute to the pathophysiologic impact of oxidative stress. In particular, the aldehydic end-product 4-hydroxynonenal appears as a major candidate, because it is: generated from the most abundant class of PUFAs; provided with three functional groups; lipophilic and relatively diffusable; and highly reactive with cellular macromolecules. The number of reports on the detection, reactivity, and biochemical effects of HNE steadily increases. Moreover, increased steady-state levels of the aldehyde have already been detected in a large variety of disease processes occurring in humans (Table 1), thus indicating a potential significant role of HNE in pathology (*48*, *65*, and references herein).

Still much investigation is needed to clarify the actual involvement of HNE and related aldehydes in the pathogenesis

f human diseases. In particular, related to challenging topics or future research are the following questions: (a) Does HNE regulate its own synthesis and degradation in vivo? (b) Has HNE any role in signal transduction and gene expression under normal conditions? (c) What is the HNE impact in the progression of chronic inflammatory and degenerative diseases ? Significant advancement on these questions should also contribute to suitable updating of pharmacological intervention, e.g., in neurodegenerative and atherosclerotic diseases.

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